Effect of rate and extent of starch digestion on performance, physiology and behaviour of broilers and laying hens

A Thesis Submitted to the College of Graduate and Postdoctoral Studies in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, SK Canada

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Abstract

The effect of rate and extent of starch digestion on broiler and laying hen performance, digestive tract physiology and feeding behaviour, with particular focus on the ileal brake activation, was assessed. Semi-purified wheat (WS, rapidly digested) and pea (PS, slowly digested) starch were combined to create six WS:PS ratios (100:0, 80:20, 60:40, 40:60, 20:80, 0:100) in treatment diets, and were fed to Ross 308 broilers for 28 days and Lohmann LSL-lite laying hens for 20 weeks. Mortality-corrected gain:feed ratio of broilers was maximized at 25% PS. Breast meat yield relative to live body weight increased linearly with dietary PS inclusion, while fat pad, and breast and thigh skin decreased in a linear fashion. Overall hen-day egg production increased linearly with PS, but it was maximized at 70% PS during the second half of the experiment. Feed:egg mass ratio was minimized at 26% PS (quadratic). Ileal brake activation potential was found in both broilers and laying hens. Increasing PS inclusion in broiler diets resulted in lower *in-vivo* starch digestibility, and quadratic responses in both crop pH (minimum at 55% PS) and ileum SCFA (maximum at 58% PS). Likewise, crop and ileum pH in laying hens increased with PS inclusion. Actual indications of ileal brake activation were not as clear. While most digestive tract morphological parameters increased linearly with PS in broilers, GLP-1 and PYY serum concentrations and small intestine transcript abundance were not affected by PS inclusion. Feeding behaviour of broilers was not affected either. Digestive tract parameters of laying hens responded with a combination of linear increasing and quadratic effects with maximum values in the mid-range of PS concentrations. In addition, serum GLP-1 also increased linearly, while PYY was maximized at 34% PS. However, dietary PS concentration did not affect feed passage rate. Likewise, laying hen day-time feeding behaviour was not affected by PS concentration, but night feeding behaviour increased with PS inclusion. In conclusion, the positive effect of including PS in poultry diets was confirmed, but L-cell activation and its consequences seem to differ between bird types and act in a different manner compared to mammals.

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List of Abbreviations

AgRP	agouti-related peptide
AME	apparent metabolizable energy
BW	body weight
BWG	body weight gain
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
cm	centimeter
CP	crude protein
d	day(s)
D	diet
DBL	drinking bout length
DF	dietary fibre
DM	dry matter
F:G	feed-to-gain
$F:G^m$	mortality corrected feed-to-gain
FBL	feeding bout length
FI	feed intake
g	gram(s)
G	gender
$G:F^m$	mortality corrected gain-to-feed
GLP-1	glucagon-like peptide 1
GLP-2	glucagon-like peptide 2

FCR	feed conversion ratio
FSH	folicule-stimulating hormone
h	hour(s)
HCl	hydrochloric acid
HDP	hen-day production
HHP	hen-housed production
K	potassium
kg	kilogram(s)
L	linear
LH	luteinizing hormone
Lys	lysine
Mort.	mortality
N	Number of feeding or drinking bouts
Na	sodium
NEFA	non-esterified fatty acids
NPY	neuropeptide Y
NS	not significant
OXM	oxyntomodulin
POMC	pro-opiomelanocortin
PC	pea-corn
PS	pea starch
PYY	peptide tyrosine-tyrosine
Q	quadratic

RDS	rapidly digestive starch
RS	resistant starch
SCFA	short-chain fatty acids
SDS	slowly digestive starch
SEM	standard error of the mean
SI	small intestine
T1NFB	time to first night feeding bout
TBFB	time between feeding bouts
TBNFB	time between night feeding bouts
TC	tapioca-corn
TDT	total drinking time
TFT	total feeding time
TNFT	total night feeding time
TS	total starch
wk	week(s)
WS	wheat starch

1.0 General Introduction

Due to the production demands of commercial laying hens and broilers, high-energy is an essential requirement in poultry diets. Starch is a key ingredient and principal energy source in poultry diets, and is primarily supplied by various grains. Although chickens have shown a remarkable capacity for starch digestion (Svihus, 2014), physicochemical variability in starch composition and structure between plant sources results in digestibility differences (Weurding et al., 2001). In 1992, Englyst et al. developed a classification method based on *in vitro* digestibility assays which mimicked human digestion conditions for a variety of starch sources; it resulted in starch being classified as rapidly, slowly or not digested (resistant starch). The same concept can be applied to poultry to compare starch sources based on their *in vivo* or *vitro* digestion characteristics. Rapidly and slowly digested labels can be applied in relative terms to compare two or more starch sources, while resistant starch would be undigested starch present in the terminal ileum.

Research has shown that starch digested slowly and to a lesser extent can affect broiler feed efficiency in a positive way (Weurding et al., 2003; Gutiérrez del Alamo et al., 2009). Weurding et al. (2003) found a 2.0% improvement in broiler feed efficiency when feeding a pea-corn as compared to a tapioca-corn based diet. *In vitro* analysis showed that the former was more slowly digested that the latter. Similar findings were reported by Gutierrez del Alamo et al. (2009) when feeding diets with variable starch digestion rates. In that study, it was found that feed efficiency was improved by 1.3% when digestion rate was reduced by 14.3%. However, even lower digestion rates resulted in poorer feed efficiency. A potential criticism of these studies was the use of intact starch sources, which can result in confounding effects of other grain components. Provided that starch digestibility characteristics are maintained, the use of semi-purified starch sources may avoid this problem.

Although starch digestion rate affects feed efficiency in poultry, little is known about the responsible mechanisms. Starch digestion rate and extent can affect a number of physiological mechanisms and conditions, such as post-prandial glucose regulation and metabolism, bacterial fermentation, as well as direct and indirect effects on the small intestine. The glycaemic index is the increase in blood glucose concentration during and after a meal, which is the resulting balance between the rate of glucose absorption from the digestive tract and the action of insulin

(Jenkins et al., 1981). A high correlation can be found between starch digestion rate and the glycaemic index, where more rapidly digested starches have higher glycaemic indexes (Holt et al., 1992), and therefore have a larger effect on glucose metabolism. The presence of starch or glucose in distal sections of the small intestine it has been postulated to provide an energy source to the local enterocytes, and replace amino acids for this purpose (Wu et al., 1995; Enting et al., 2005). Starch present in the digestive tract, particularly in distal small intestine, caeca and colon, can also be utilized as a prebiotic, affecting the microbiome and chicken resistance to infection (Regmi et al., 2011b). Likewise, nutrient sensing cells can be activated by glucose and its fermentation products, short chain fatty acids (Furness et al., 2013). L-cells are a type of nutrient sensing cell whose prevalence increases in the distal small intestine, and can release glucagon-like peptide-1 (GLP-1) and peptide tyrosine tyrosine (PYY) in response to meal size, fat and carbohydrates (Janssen and Depoortere, 2013). These neuropeptides can reduce gastric emptying and increase satiety (van Avesaat et al., 2015), thereby affecting digestion dynamics and feeding behaviour. In summary, utilization of more slowly digested starch results in gradual glucose absorption that requires less regulation by insulin, and may more closely match an animal's physiological energy requirement, allowing the immediate utilization of glucose for processes such as muscle deposition instead of energy storage (Deep, 2018). Also, slowly digested starch (SDS) fermentation products may aid intestine barrier function, thereby reducing the need for immune response activation.

Although there appear to be benefits due to the incorporation of more SDS into poultry diets, this effect needs to be verified with the use of semi-purified starch sources. In addition, research is needed to determine the mechanisms behind their effects. The general objective of this project is to determine the effects of starch digestibility rate and extent on the performance, physiology and behavior of broiler chickens and laying hens using semi-purified starch sources. The general hypothesis is that a more slowly and/or poorly digested starch source, such as pea starch, will result in stimulation of the ileal brake, and thereby affect digesta passage rate, feed intake, digestive tract size and bird behavior. In addition, dietary pea starch, by activating the ileal brake, will extend the time feed is present in the digestive tract of broiler chickens after feed withdrawal.

2.0 Literature Review

2.1 Starch

Starch, the most important source of energy in poultry diets, is the principal storage polysaccharide in plants, and it can be found in the chloroplasts (green leaves) and amyloplasts (seeds, pulses and tubers) (Ellis et al., 1998; Sajilata et al., 2006). Its empirical hydrated formula is ($C_6H_{10}O_5 . H_20$) and it is composed predominantly of amylose and amylopectin, two polymers of D-glucose, with the ratio varying among plant sources (Sajilata et al., 2006). Starch molecules in their native state are very complex and large, and although their structure is not completely understood, crystalline and amorphous layers are alternated to form rigid semi-crystalline granules, with the final architecture structure varying across plant sources (Smith, 2001; Svihus, 2014). The end product of starch digestion is glucose, the main energy source used for metabolic processes in animals, but physicochemical characteristics of starch granules affect their rate and extent of digestion.

2.1.1 Chemical structure

Starch is a homopolymere of glucose molecules connected by 1-4 and 1-6 α-glycosidic bonds (Asp, 1996). Although most starches are composed of 20-25% amylose, this amount varies among sources, affecting its digestibility (Parker and Ring, 2001). For instance, while wheat starch granules are indeed usually comprised of 20-25% amylose, pea starch has a higher amylose content ranging from 33 to 50% (Ratnayake et al., 2002), whereas others, known as waxy starches, have very low amounts of amylose (Jane, 2006).

Amylose is a relatively short molecule (100 kDa), 99% linear, with α(1-4) linkages, that forms double and single helices in its native state (Buléon et al., 1998). In the interior of these helices, it is common to find other compounds such as iodine, alcohols and fatty acids (Svihus, 2014). Amylopectin, the second molecule composing starch, is a much larger molecule (10^4 to 10^6 kDa), heavily branched (approximately once every 20 glucose units) and with approximately 95% α(1-4) and 5% α(1-6) linkages (Biliaderis, 1991).

2.1.2 Physical structure

Starch is found in granules of various shapes (spherical, oval, polygonal, dome-shape, elongated, etc.) and sizes (<1-100 microns) depending on the source (Jane, 2006). Each granule has a center of growth or hilum from which multiple concentric layers can be observed. These layers are formed by the radial arrangement of amylose and amylopectin molecules linked by hydrogen bonds (Parada and Aguilera, 2011). Each ring has low and high-density regions. The latter have a crystalline portion formed by packed amylopectin chains and an amorphous region composed of amylopectin branches and amylose molecules disposed in a disorganized fashion. Amylose molecules are the main component of low-density regions (Jacobs and Delcour, 1998; Copeland et al., 2009).

Both the degree and arrangement of the crystallites can vary among starches. Crystallites can be arranged into an A, B or C-type. The differences are in how the double helices are packed and the water content in between them. A-type starch is formed by shorter chains that are arranged in monoclinic unit cells where water is distributed around each cell. B-type starch contains long chains arranged into hexagonal unit cells that are more packed together and the water content is concentrated in the center of 6 units. C-type is a combination of A and B-types (Jane, 2006).

2.1.3 Starch digestion

All ingested material goes through a lubricating, mechanical, chemical and bacterial treatment as it passes through the digestive tract. In most vertebrates, ingested material is first mechanically broken, while saliva moisturizes and starts chemical digestion. Among other components, the saliva often contains amylase, an enzyme that can initiate starch digestion. The presence of taste buds in the oral cavity aids in the detection of food items and in the avoidance of harmful ones. Once swallowed, the esophagus conducts the bolus to the stomach where acid chemical digestion, particularly of protein present in the bolus, takes place. In the midgut, acid is neutralized and major chemical digestion of protein, fats and carbohydrates occurs. Secretions from the duodenum, pancreas and liver aid in this process. In this section, pancreatic α-amylase continues the starch digestion, while final digestion occurs along the small intestine with the help of brush-border enzymes. The resulting monosaccharides (including glucose molecules), along

with amino acids, small peptides and free fatty acids are absorbed by the enterocytes to fuel the body. Any undigested material, including carbohydrates, is exposed to the fermenting action of bacteria along the digestive tract, although particularly prominent in the distal small intestine and large intestine. Once the cloaca is reached and after water and ions are absorbed, undigested material along with any other body waste is expelled through the anus.

2.1.4 Factors affecting digestion

The digestibility of the starch molecule can vary depending on certain characteristics of the molecule, including its degree of crystallinity, the size of the granules, the amylose:amylopectin ratio, the presence of lipids, proteins and other polysaccharides in the diet, encapsulation and other physicochemical attributes (Parada and Aguilera, 2011).

Resistance to digestion increases as B-type crystallites increase (Hoover and Zhou, 2003). B-Type starches are usually characterized by longer amylopectin chains, in addition to a non-uniform distribution of water, making access by digestive enzymes difficult. The rate of starch hydrolysis is also proportional to the surface area of the granules (Noda et al., 2005). However, hydrolysis occurs from inside out, so also the number and size of surface pores is an important factor (Zhang et al., 2006).

The amylose:amylopectin ratio is a main factor affecting digestibility, with higher proportions of amylose associated with lower digestibility (Behall, 1988; Lehmann and Robin, 2007). During starch synthesis, is not uncommon for starch-lipid associations to be formed (Tester et al., 2004). However, the presence of these lipids reduces the contact area for hydrolytic enzymes. Moreover, as the amount of lipid increases, swelling of the granules decreases due to the increased hydrophobicity, also reducing digestibility as enzymes require water for enzymatic degradation (Jane, 2006).

Processing of starch can also affect its digestibility. Factors such as particle size reduction (e.g. grinding) and heat processing (e.g. pelleting) are of primary importance. Reducing particle size is associated with increased surface area for α-amylase action and therefore an increased rate and extent of starch digestibility. Another potential influence is the gelatinization process, which increases starch digestibility. When native starch, in the presence of water, is exposed to high

temperatures, the granular structure disintegrates. This makes starch more easily available to the activity of α-amylase. Gelatinization temperatures for most cereal grains range between 50-70°C. However, during the normal pelleting process, the limited moisture content of the granules results only in a small amount of gelatinization (Svihus, 2014). In relation with gelatinization, there is also retrogradation of starch upon cooling of the gelatinized starch; meaning there is a full or partial recrystallization of the structure, which decreases digestibility (Wu et al., 2009).

2.1.5 Kinetics

Starch digestion is mostly carried out by pancreatic α-amylase. Because α-1,6 amylopectin branch linkages cannot be hydrolyzed, α-1,4 links adjacent to the branching point cannot be hydrolyzed either due to steric inaccessibility (Singh et al., 2010)

Starch hydrolysis has been proposed to follow a first order equation (Goñi et al., 1997):

$$C - C_{\infty} (1-e^{-kt})$$

Where C is the concentration of starch hydrolyzed at time t, C_{∞} is the equilibrium concentration, k is the kinetic constant and t is the chosen time. Using this equation, Goñi et al., found that legumes had a lower digestion rate and extent when compared to cereals or processed foods.

2.1.6 Starch digestion in poultry

The avian digestive tract is very similar to that of other monogastric animals, although smaller due to the requirement for flight. Despite the relatively small digestive tract, the starch digestion capacity of chickens is very high, reaching values close to 100%, and being above that of humans and pigs for instance, even at young ages (Zelenka and Ceresnakova, 2005). This is believed to be related to a particular high secretion of amylolytic enzymes in the duodenum. Although starch digestibility is overall high, Thomas et al. (2008) found that digestibility tends to be reduced from day four or five and until seven days of age, to be completely restored by 14 days.

In order for starch to be absorbed, feed is ingested and sometimes stored in the crop, a thin-walled expansion of the esophagus. If food is stored in the crop, it can undergo digestion, with the extent related to storage duration. Digestion occurs mostly as a result of fermentation by lactobacilli, which are the predominant bacteria in this organ. Moistening may also activate enzymes present in the feed (Onyango et al., 2005). When the proventriculus and gizzard are empty, chemical and mechanical stomachs respectively, feed coming from the esophagus or the crop storage is released into them. In the proventriculus and gizzard, feed undergoes chemical and physical digestion. Secretions such as HCl and the zymogen pepsinogen (converted to the active protease pepsin) from the proventriculus initiate the chemical digestion process, if it hasn't started already in the crop. Once in the gizzard, feed is physically ground to smaller particles thanks to its powerful muscles and sand-paper-like koilin surface. How long feed is retained in this section of the digestive tract is related to particle size, with large particles retained longer (Hetland and Svihus, 2001; Svihus, 2014). In addition, there is some suggestion that larger gizzard sizes might be associated with higher starch digestibility (Hetland et al., 2002). This is speculated to be due the reduction in particle size increasing surface area for action of pancreatic α-amylase in the small intestine. It may also reduce encapsulation of starch granules by protein.

Once particles are small enough, they are released into the small intestine, which is where most of the digestion and absorption of nutrients occur, including starch, in a manner similar to other monogastric animals. In the duodenal loop the acidic contents from the gizzard are mixed with the bile and pancreatic juices secreted at the end of the duodenum, increasing the pH of the digesta. Here is where α-amylase breaks down starch mainly into maltose and the higher dextrin isomaltose (Annison and Topping, 1994). To do this, it has been shown that digestion starts at surface pores and interior channels, allowing amylase to enter the molecule and digest the granule from the interior out (Zhang et al., 2006; Svihus, 2014). Enterocyte membrane associated maltase and isomaltase split maltose and isomaltose into glucose (Gray, 1992). Glucose molecules can be absorbed directly through the intestinal mucosa by a glycoprotein that co-transports 2 molecules of Na^+ along with glucose (SGLT-1) (Pencek et al., 2002; Zhang and Hamaker, 2009). The Na^+ gradient necessary for this transport to the inside of the cell is provided by the Na-K ATPase, which constantly pumps Na^+ outside the cell.

The intestinal mucosa uses about 30% of the ingested glucose for cell maintenance, converting it into lactate, while sending the remaining glucose by systemic blood to the rest of the body. In the case of diets containing only RDS, no glucose reaches the ileum to be absorbed as starch is completely digested and absorbed by the end of the jejunum (Riesenfeld et al., 1980). Thus, the ileum must rely on receiving glucose through the systemic blood stream. Alternatively, gut tissues are able to use glutamine as an energy provider, and so when all starch is digested in the upper small intestine, more amino acids may be oxidized to cope with energy demands (Weurding et al., 2003). The starch that is not digested along the digestive tract can be fermented by the bacteria present in the small intestine, but particularly in the caeca and colon, resulting in the production of short chain fatty acids (SCFA). These fermentation products provide a supplemental energy source that can also be absorbed, having a positive effect on the epithelium of the distal digestive tract, or can be utilized by bacteria (Parada and Aguilera, 2011). Production of SCFA has also been shown to increase barrier function and the secretion of hormones GLP-1 and PYY, that result in reduced intestinal tract motility among other effects (Choct, 1996; Massimino et al., 1998; Zhang and Hamaker, 2009).

2.1.7 Nutritional classification of starch

Englyst et al. (1992) developed an *in vitro* digestion method of starch that mimics human starch digestion. After testing a variety of starch sources, they classified sources according to how fast they were digested. This classification defines RDS as any starch digested in 20 minutes or less, SDS to starch digested in the period between 20 minutes and 2 hours and resistant starch (RS) to any remaining undigested fraction after 2 hours (Lehmann and Robin, 2007). This technique has been adapted to more accurately reflect the chicken digestive tract (Karunaratne et al., 2018).

When this classification is applied to *in vivo* digestion, the fraction that is the easiest to digest, known as RDS, is completely digested and absorbed by the end of the jejunum. This results in a rapid increase of blood glucose, usually followed by a subsequent episode of hypoglycemia, generating high stress on regulatory systems of glucose homeostasis (Ludwig, 2002; Zhang and Hamaker, 2009). Starch would be considered SDS when it is still completely digested, but its final digestion occurs in the ileum, resulting in a slow and prolonged release and

absorption of glucose. Finally, RS is undigested, and therefore reaches the large intestine where it can be fermented by the microflora. Fermentation of starch along with non-starch polysaccharides produces SCFA, which are absorbed and provide an additional source of energy (Englyst and Hudson, 1996). Resistant starch has been defined as "the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals" (Asp, 1996; Weurding et al., 2001).

2.1.8 Slowly digested starch

Slowly digested starch is any type of starch that due to its physicochemical characteristics is digested more slowly, and so it reaches the distal ileum before it is completely digested. The rate and extent of digestion of starch is reflected by the magnitude and duration of the glycaemic response after a meal (Englyst and Hudson, 1996). Slowly digested starch results in a slower and gradual release of glucose, resulting in a moderate post-prandial glycaemic and insulinemic response. These characteristics have promoted a considerable amount of research regarding the effect of increasing SDS intake in humans, since it has been found to have a positive effect in treatment of common chronic diseases such as diabetes, obesity and cardiovascular disease, in addition to improving satiety and mental performance (e.g. Behall, 1988; Cummings and Englyst, 1995; Liu et al., 2000; Seal et al., 2003; Alviña and Araya, 2004).

An abnormally high blood glucose concentration, which can be induced as a consequence of RDS consumption over an extended period of time, may damage cells, tissues and organs. Some mechanisms worth mentioning are the production of free radicals in the mitochondria that can damage cell membranes and DNA, and insulin resistance due to the continuous activation of β-pancreatic cells (Zhang and Hamaker, 2009). In addition, significantly faster and greater changes in blood glucose, insulin and non-esterified fatty acid (NEFA) concentrations occur in humans after a meal rich in RDS compared to SDS. Diets high in SDS tend to produce the opposite effect, reducing oxidative stress and obesity, while increasing glycemic control, which results in decreased glucose peaks and episodes of hypoglycemia, improved lipid response and increased insulin sensitivity. These characteristics show the potential benefits of SDS for the management of metabolic syndrome and diabetes (Lehmann and Robin, 2007). Glucose is the main fuel for the brain and so glucose concentration may influence brain performance; it has

been suggested that a SDS diet benefits brain performance. However, evidence supporting this is inconsistent (Lehmann and Robin, 2007). There is evidence that consumption of SDS induces a sustained elevation of GLP-1 and PYY, two hormones released in the distal small intestine and large intestine, in the later stage of digestion, which can decrease gastric emptying, and increase satiety (Wachters-Hagedoorn et al., 2006; Zhang and Hamaker, 2009).

2.2 Glucose metabolism and glycemic index

The main use of glucose once it is absorbed is energy production through cellular oxidation, in addition to synthesis of glycogen, fatty acids, nonessential amino acids, vitamin C and other metabolites (Braun and Sweazea, 2008). When the bird is eating, the rapid absorption of glucose results in a glycemic peak. The height of the peak is related to the nature of the starch source, and high-glycemic index starchy foods are more insulinogenic (Björck et al., 2000).

The glycemic index is the comparison of the area under the response curve of insulin between a test ingredient or meal and a reference food such as white bread or glucose when they are fed in the same amount (Jenkins et al., 1981). Thus, food can be classified as having a low glycemic index when this number is equal or under 55 or high when it is 70 or more. When comparing meal fed individuals, RDS meals result in higher glycemic peaks than when eating a meal with SDS. Thus, RDS foods have high glycemic indexes, and since the amount of glucose absorbed exceeds the current needs of the body, the surplus of glucose is stored in the form of glycogen or fat, a process that consumes energy. However, as the body processes consume the available glucose, blood sugar concentration diminishes and activates catabolism, starting with glycogen (Mayer, 1996). As these stores decrease due to the high metabolic rates of birds, stimulation of gluconeogenesis from fat and amino acids begins (Scanes, 2009). However, according to Dupont et al. (2008), catabolism in the liver is activated only after five hours of fasting in chickens, which is when proteolysis is induced. Therefore, there should not be a high amount of catabolism in *ad libitum* fed chickens, with the exception of dark periods longer than five hours.

A continuous glucose supply, like the one resulting from SDS diets, would result in a gradual insulin release and may lead to a more efficient utilization of amino acids because

glucogenic energy should be available for protein deposition to occur (Weurding et al., 2001). In addition, as glucose concentration does not change as dramatically as with RDS meals, less regulation would be required, including less energy spent storing the surplus glucose since it can be utilized as it is absorbed and for a longer period of time than RDS.

In addition to the characteristics of the starch itself, other components within the food matrix may influence glucose metabolism and glycemic index (Singh et al., 2010). For instance, the presence of proteins produce a higher insulin response, resulting in a faster absorption of peripheral glucose, lowering post-prandial blood glucose concentration. On the other hand, glycemic index can be reduced as a consequence of proteins surrounding the starch granules, reducing starch digestibility (Hamaker and Bugusu, 2003). Fat, through a reduction of gastric emptying also reduces post-prandial glycaemia (Mateos et al., 1982). An analogous situation is that of soluble fibre, which reduces gastric emptying by increasing the viscosity of the gut contents (Lehmann and Robin, 2007).

2.3 Starch Fermentation

Starch is not only subjected to digestion, but can also be fermented by digestive tract bacteria. The bacterial community composition is variable, providing an ability to respond to changing environmental conditions including diet composition. According to Apajalahti et al. (2004), around 640 species of bacteria belonging to 140 genera are present in the chicken digestive tract. The highest number of bacteria are present in the caeca (10^{10}-10^{11}/g), while the small intestine follows close behind (10^{8}-10^{9}/g; Yeoman et al., 2012).

Starch fermentation by the microbiome produces gases such as hydrogen and methane as well as SCFA, mainly butyrate, acetate and propionate. Short chain fatty acids reduce the pH of the luminal contents which inhibits proliferation of some bacteria such as *Salmonella spp*. and *C. perfringens* (Macfarlane and Macfarlane, 2003). In addition, butyrate is a preferred energy substrate for distal digestive tract enterocytes and it is believed to improve barrier function by inhibiting transcription of NFkB and stimulating mucin and antimicrobial peptide production (Eeckhaut et al., 2011).

Once absorbed, butyric acid is mostly metabolized to produce energy in the enterocyte, while propionic acid and acetic acid reach the portal vein (Vipperla and O'Keefe, 2012). The main fate of propionic acid is as a substrate for gluconeogenesis in adipose tissue and the liver, while acetic acid serves as a substrate for cholesterol synthesis in liver, muscle and other peripheral tissues (Vipperla and O'Keefe, 2012).

2.4 Satiety

Hunger is the word used to describe the feeling of discomfort or weakness caused by the lack of food, coupled with the desire to eat. Research conducted in humans has demonstrated that it is triggered by a combination of internal signals such as the loss of energy stores (fat and glycogen) and low blood glucose, and external signals such as the presence of food. Satiety, on the other hand is the exact opposite state to hunger. It refers to the processes that result in feed intake cessation, reduced daily feed intake, and shorter feed intake bouts or longer times between feed intake bouts (Tolkamp et al., 2011). This process is initiated by the release of satiety signals from the small intestine when the presence of nutrients is sensed. These satiety signals are enteropeptides and neurotransmitters, such as PYY, GLP-1 and cholecystokinin (CCK), that inhibit hunger signals at the level of the gut and brain, resulting in a reduction in food intake (Maljaars et al., 2007). In addition, satiety can be promoted by pro-inflammatory cytokines and the concentration of nutrients such as glucose and free fatty acids in the blood (Morton et al., 2014).

Nutrient receptors can be found throughout the digestive tract, mainly present in enteroendocrine cells. Receptors directly related to glucose sensing are the sweet taste receptor, the heterodimer T1R2-T1R3 which is found in A, K and L cells, and SGLT1 which is found in the brush-border membrane of enterocytes (Furness et al., 2013). Although sequencing of chicken genome has demonstrated that chickens lack the T1R2 gene (Cheled-Shoval et al., 2015), evidence exists that chickens are able to sense and respond to carbohydrates such as sucrose, fructose and xylose (McNaughton, 1978; Gentle, 1985; Ganchrow et al., 1990). Treesukosol et al. (2009) hypothesized that only T1R3 might be necessary for sweet–taste sensing. In addition, starch fermentation products, SCFAs, can be sensed trough FFAR2 and FFAR3 receptors (Psichas et al., 2015a).

There are two regulatory systems of feed intake, short-term or peripheral satiety system, which is meal-related, and long-term, which is related to body energy stores (Richards et al., 2003). As soon as feed is ingested, a number of satiating signals arise including the activation of tension and mechanoreceptors and the release of peptide hormones (Näslund and Hellström, 2007). Many of these signals can rapidly reach the brain through vagal afferents (Roura et al., 2008). The hypothalamus then integrates all signals coming from the blood metabolites, digestive tract, reserve tissues and nervous system signals, resulting in the activation or inhibition of hypothalamic neuropeptides in order to maintain homeostasis. Signals indicating a low level of metabolites and depleted reserves activate neuropeptide Y (NPY), agouti-related protein (AgRP) and GABA neurons which stimulate food intake. Signals indicating the opposite state activate the infundibular pro-opiomelanocortin (POMC), and cocaine and amphetamine-related transcript (CART), which have anorexigenic properties (Song et al., 2013).

Because it is unlikely that there is much variation in the concentration of plasma nutrients in chickens fed balanced diets on an ad-libitum basis, depleting stores should not play a significant role in the control of meal-eating, except possibly at the end of the dark period if it is longer than four hours. Thus, diurnal hunger and satiety are probably driven by a combination of emptying and filling parts of the digestive tract and nutrient sensing (Savory, 1999). Richardson (1970) found that the amount of digestive contents in the small intestine do not vary much, although there might be some variation in the crop, proventriculus and gizzard suggesting that meal initiation might be related to partial gizzard emptying (Savory, 1980; Savory and Hodgkiss, 1984). In addition, transit time seems to be another factor that could affect feeding behaviour since there is evidence that as transit time shortens (becomes faster) the interval between meals shortens as well (Savory, 1980; Savory and Hodgkiss, 1984). This might or might not affect feed intake.

2.5 Regulation of feed intake in poultry

The presence of feed in the gastrointestinal tract stimulates the release of the number of peptides that regulate gut motility, secretions, and satiety in the brain. Although digestion and feed regulation in poultry is very similar to mammals, there are some differences. Like in

mammals, the hypothalamus receives all signals related to energy balance status via the vagus nerve and blood. To process the appropriate response, either satiety or hunger mechanisms are activated (Boswell, 2005; Richards and Proszkowiec-Weglarz, 2007; Figure. 2.1).

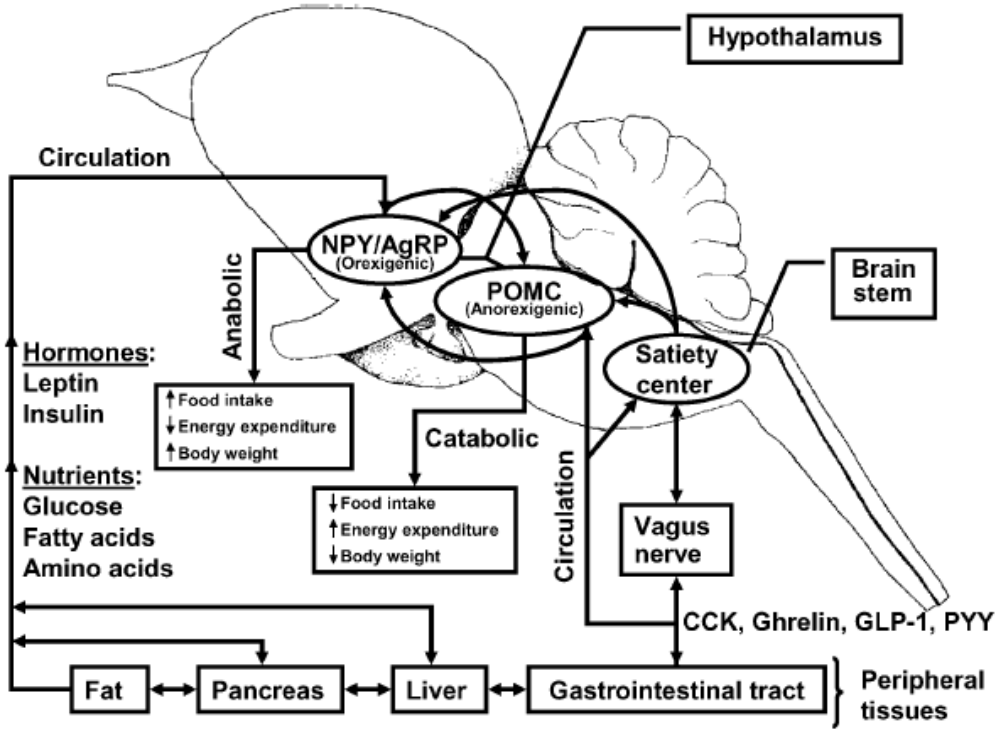

Figure 2.1. Proposed model describing long-term appetite regulation in poultry. Extracted from Richard and Proskawiec-Weglarz (2007). NPY = neuropeptide Y; AgRP = agouti-related peptide; POMC = proopiomelanocortin; CCK = cholescystokinin.

2.5.1 Ghrelin

Ghrelin is a good example of the hormone that produces opposite effects in mammals and chickens. In mammals, this stomach secreted hormone stimulates feed intake. Intra-cerebro-ventricular injections as well as ghrelin infusions have shown to increase feed intake both in rats and humans respectively (Nakazato et al., 2001; Wren et al., 2001). However, although dieting increases ghrelin plasma levels, gastric bypass surgery impairs its secretion, suppressing its plasma levels (Cummings et al., 2002).

Ghrelin is a 26-long amino acid hormone that shares 50% total sequence identity to the human counterpart. The mRNA expression of ghrelin receptor has been detected in central nervous tissues such as the pituitary, hypothalamus, telencephalon, cerebellum and brainstem, as well as the ovary, kidney, liver and digestive tract (Kitazawa et al., 2013). Chicken ghrelin is secreted from the proventriculus, and its levels increase after fasting (Kaiya et al., 2007). However, it has shown to have an inhibitory effect on feed intake (Richards and Proszkowiec-Weglarz, 2007; Kaiya et al., 2009). Its effect on feed intake seems to be mediated by the corticotropin-releasing factor instead of the NPY-orexin system like in mammals (Saito et al., 2005). Like in mammals, ghrelin is also involved in digestive tract contractile activity. Its effects decreases from crop to duodenum to increase after (Kitazawa et al., 2007, 2009).

2.5.2 Leptin

Leptin, another anorexic peptide in chickens, has been proposed to reduce feed intake through the same mechanism as found in mammals, which is by inhibiting NPY expression in the hypothalamus (Decuypere and Buyse, 2005). However, it has been suggested that the responsiveness to leptin differs between laying hens and broilers. Chickens with lower growth rates such as layers, may be more responsive to leptin levels than those with high growth rates, including broilers (Richards and Proszkowiec-Weglarz, 2007).

2.5.3 Cholecystokinin

CCK is a potent inhibitor of feeding, and is secreted from the small intestinal I cells in response of feed ingestion. It appears to function in a similar fashion in both chickens and mammals (Owayang and Heldsinger, 2011). This peptide stimulates gastric emptying and the release of pancreatic enzymes, while depressing appetite in the brain (Richards, 2002). However, cholecystokinin cannot penetrate the blood brain barrier, and so it depresses appetite in the brain via vagal afferent fibers (Owayang and Heldsinger, 2011).

2.5.4 Peptide tyrosine-tyrosine

PYY is a hormone that is produced by the L cells from the duodenum to the distal ileum of chickens (Park and Bloom, 2004; Singh et al., 2012). Blood concentrations of this hormone have been found to increase after feeding (Stadlbauer et al., 2013). PYY slows down gastric emptying and intestinal transit when the distal sections of the small intestine sense unabsorbed nutrients, in particular fat-rich meals (Taylor, 1993). Aoki et al. (2017a) found that feed intake changes in a dose-dependent manner when Ross 308 male chicks were injected with chicken PYY. In addition, since plasma injections of PYY do not produce the same effect as nutrient infusions, it is believed that the actions of PYY are not endocrine but paracrine through the vagus nerve (Maljaars et al., 2008).

2.5.5 Neuropeptide Y

Neuropeptide Y, is the most conserved peptide of the pancreatic polypeptide family. Knowledge regarding its physiological roles in chickens remains scarce, but like in mammals, NPY strongly stimulates feed intake and locomotor activities in chickens (Kuenzel and McMurtry, 1988; Chen et al., 2016). However its effect decreases when GLP-1, a strong inhibitor of feed intake, is centrally administered (Furuse et al., 1997).

2.5.6 Glucagon-like peptide-1

The neuroendocrine peptide GLP-1, is secreted from the L-cells of the digestive tract. In chickens, like in mammals, GLP-1 is derived from a single proglucagon gene that possesses multiple mRNA transcripts (glicentin-related peptide, glucagon, intervening peptide-1 and 2, and glucagon-like peptide-1 and 2; Richards and McMurtry, 2009). Tissue specific alternative splicing occurs to the proglucagon gene in chickens, producing two mRNA transcripts (A and B). Proglucagon B is predominant in the duodenum and likely the rest of the small intestine (Richards and McMurtry, 2008). Proglucagon mRNA expression levels in the gastrointestinal track of 8 day-old chicks were detected in proventriculus, jejunum and ileum (Honda et al., 2017). Studies in humans indicate that GLP-1 is secreted upon the arrival of nutrients, but it seems that fats and carbohydrates are more potent stimulators than protein (Burrin et al., 2003). In particular within the carbohydrates, those transported via the sodium-glucose co-transporter

are good promoters of this neuropeptide (Burrin et al., 2003). In addition, both dietary fiber and SCFA increase GLP-1 levels. Finally, its secretion is also mediated by endocrinal and neural control, since circulating levels of GLP-1 increase before the arrival of nutrients to the distal digestive tract in humans. This is believed to be in response to the increasing levels of gastric inhibitory polypeptide (GIP) secreted by K cells in the proximal small intestine when nutrients are sensed; this signal is transmitted via the vagus nerve to the distal digestive tract (Burrin et al., 2003). The physiological importance of GLP-1 in chicken satiety has yet to be determined.

2.5.7 Oxyntomodulin

Oxyntomodulin (OXM), another of the enteroglucagon peptides, shares some similarities with GLP-1, including being secreted by intestinal L cells. It exerts its effect through the activation of the GLP-1 receptor as its lacks specific receptors, although its affinity is lower when compared to GLP-1 (Stanley et al., 2004).

2.6 Ileal brake

As previously stated, the presence of nutrients in the digestive tract results in signals that enhance satiety, inhibit feed intake, and slow gastric emptying and digestion rate. This inhibition comes from both proximal and distal sections of the small intestine; however, the feedback is stronger when it comes from the ileum (Meyer et al., 1998b) and it is known as the ileal brake. The action of the brake is dose-dependent, and three enteropeptides are believed to mediate its actions: GLP-1, PYY and OXM (Maljaars et al., 2008).

The secretion of these peptides in response to the arrival of nutrients to the distal sections of the digestive tract results in delayed gastric emptying, secretion and motility (Stanley et al., 2004). The delay in gastric emptying is associated with a decrease in the frequency of peristaltic waves and an increase in the pyloric sphincter pressure. Likewise, jejunal contractions are also reduced, which altogether increase transit time (Maljaars et al., 2008). Increased plasma concentrations of GLP-1, OXM and PYY have been shown to correlate with post-prandial satiety, at least in mammals (Zhou et al., 2008). Secretion of GLP-1 starts even before the nutrients arrive to the distal digestive tract, which suggests the involvement of both endocrine

and neural signals. It is the stimulation of GIP secretion from the K-cells of the duodenum, as well as the vagus nerve, at the arrival of digestive contents, which stimulates GLP-1 release from the L-cells of the distal digestive tract (Burrin et al., 2003).

Gee et al. (1996) found that products of carbohydrate fermentation, SCFA, could promote proglucagon derived peptide secretion from L cells in rats. This was associated with an acidification of the caecal contents. Similar results were obtained in rabbits, where the concentration of PYY was increased by physiological concentrations of n-butyrate and acetate (Longo et al., 1991). Diets high in fiber result in colon and caecal fermentation producing SCFA. Several articles report an increase in post-prandial concentrations of PYY and GLP-1 in animals fed fibrous diets (e.g. Keenan et al., 2006; Zhou et al., 2008; Bosch et al., 2009; Singh et al., 2012; Brooks et al., 2017). It is believed that the production of SCFA is a powerful stimulus for the secretion of PYY and GLP-1 by L-cells (Bosch et al., 2009). In summary, a substantial amount of research has been done regarding L cells and their activation in mammals. However, little is known about them in non-mammalian species such as chickens.

2.7 Feeding behaviour

Feed intake is not only affected by energy status and the filling of different sections of the digestive tract, but also by experience, sensory stimuli, and the behaviour of conspecifics. Chickens have an innate exploratory behaviour that is developed soon after hatch (Rogers, 1995). The tip of the beak is highly innervated and helps in the manipulation and identification of edible items (Rogers, 1995), along with sight and taste senses (Roura et al., 2008; Dey et al., 2017). This is accompanied by the development of a hunger-nutritional reward system, and increasing pecking of preferred objects (Rogers, 1995). The presence of other chickens shortens the identification process and increases feeding behaviour through social facilitation (Keeling and Hurnik, 1996). All this indicates that although energy demands and gut capacity are important modulators of feeding behaviour, many sensory and social stimuli can affect feeding behaviour as well.

2.8 Feed intake and behaviour in broilers and laying hens

Selection for increased body weight has resulted in more time resting and less time standing and foraging in the broiler behaviour repertoire when compared to laying hens (Nielsen, 2004). Interestingly, although broilers are motivated to eat, this behaviour comprises only 5-10% of their time, while most of the time is spent lying (Weeks et al., 2000). However, broilers are able to eat to near full capacity, which was proposed to be possible due to a diminution of brain satiety mechanisms by Burkhart et al. in 1983. Behavioural analysis has shown that there is a positive correlation between the length of a feeding bout and the time since the last feeding bout, suggesting that feeding behaviour is driven by hunger instead of satiety (Forbes, 2000). This view was later criticized when Bokkers and Koene (2003) found that broiler feeding behaviour was controlled by satiety if pre and postprandrial correlations were analyzed. According to these authors, broiler hunger level doesn't change substantially, but broilers will stop eating when maximal physical capacity is reached.

Despite the findings in the above paragraph, broiler feeding behaviour and feed intake can be modulated by a variety of factors including diet composition and processing, and barn management (Neves et al., 2014; Classen et al., 2017). High individual variability is reflected by total feeding behaviour accounting from one to two and a half hours daily (Weeks et al., 2000; Shynkaruk, 2017). Age is another important factor. Broilers reduce visits to the feeder as they age, but increase the length of each visit to compensate for increasing energy requirements (Weeks et al., 2000; Shynkaruk, 2017).

Although the laying hen behaviour repertoire is more variable than the one observed in broilers, feeding behaviour, defined as the time spent at the feeder, accounts for 15-23% of laying hen daily activities. This percentage tends to be higher in environments, such as conventional cages, that lack stimuli to promote other behaviours. However, there is no increase in feed intake in these situations, suggesting more time is spent foraging and manipulating feed without consumption (Tanaka and Hurnik, 1992). In the study previously mentioned, Bokkers and Koene (2003) also evaluated pre and postprandrial correlations in laying hen feeding behaviour and found that unlike broilers, laying hen feeding behaviour is equally controlled by hunger and satiety.

2.8.1 Starch type and feeding behaviour

To my knowledge, no research has been conducted on the effects of SDS diets on chicken behaviour. However, it is important to realise that the classification of starch into RDS, SDS and RS according to the Englyst et al. (1992) procedure can be somewhat arbitrary, and it is common for all fractions to be present in different proportions within a starch source. As a consequence, when working with SDS, there can be confounding effects of RS. Dietary fiber can also be a confounding factor, and both RS and fiber have physiological effects that are similar to those of SDS.

Many animals have a high motivation to forage that cannot be satisfied, especially under restricted feeding. In these cases, it is normal to find the development of stereotypic behaviour (Mason and Rushen, 2006). Research in mammals has shown that high fiber diets reduce stereotypic behaviour and increase resting behaviour in restricted fed sows (Meunier-Salaün et al., 2001). While the diluting effect of fiber in diets could explain the lower incidence of stereotypic behaviour due to longer feeding time required to compensate for dilution, this does not explain the increase in resting behaviour. In another study in sows fed sugarbeet pulp as a fermentable dietary fiber, blood glucose and insulin concentrations were more stable, and resulted in a reduction of physical activity in restricted fed sow several hours after feeding (De Leeuw et al., 2004). Similar results were found by Bolhuis et al. (2010) and Souza da Silva et al. (2014) when sows were fed diets containing fermentable starch (RS) as compared to RDS diets. Although the mechanism is not clear yet, there is increasing evidence that the microbiota produce a number of metabolites that can activate the vagus nerve, and can act as neurotransmitters and neuromodulators, with the potential to alter behaviour (Gundersen and Blendy, 2009; Macfabe et al., 2010; Cryan and Dinan, 2012; Thomas et al., 2012).

Chickens respond to fibrous diets in a similar fashion as shown by Hocking et al. (2004). A decrease in the prevalence of damaging pecking and cannibalism in broiler breeders could be seen when diets containing 200g/kg of oat hulls or 50 g/kg of sugarbeet pulp were fed to chickens compared to lower inclusion levels of oat hulls or sugarbeet pulp inclusion (Hocking et al., 2004). Nielsen et al. (2011) found that diets with 330g/kg DM of fiber increased satiety, by lowering feed intake and compensatory feeding in broiler breeders, as well as the increased the

incidence of comfort behaviours performed, when they compared a control diet with a total dietary fiber inclusion level of 165g/kg DM (with 80% insoluble fiber).

2.9 Effects of starch digestibility characteristics on performance

When comparing starch from different sources in meat-type chickens, Weurding et al. (2001) found that feedstuffs with a low starch digestion coefficient stayed longer in the small intestine. It is possible that feedstuffs with low starch digestion coefficients activate a mechanism in the digestive tract of the bird, for instance the ileal brake, which increases digesta retention time and maximizes the amount of starch being digested. They also found that although starch digestibility is generally high, it is not complete. However, no evidence of fermentation in the hindgut was found in this case. In another experiment, Weurding et al. (2003) compared two diets with different starch digestion rates, pea-corn diet (SDS) vs. tapioca-corn (RDS), on broiler chicken performance. It was found that feed efficiency improved when SDS was fed. In addition, this effect was more pronounced in diets with a low amino acid level; the authors suggested this was due to a lack of synchrony between RDS and protein digestion. Elevated insulin concentrations are required for amino acid transport and cell uptake, and the glycemic peak after an RDS meal, despite being high, does not last. According to the results obtained by Weurding et al. (2003), this situation can be solved with the utilization of some SDS in the diets of broiler chickens, which produces a longer and more steady peak in glucose, improving performance. Although in meal-fed animals this is certainly a possibility, it might not apply to broiler chickens or laying hens, which are fed *ad libitum*.

The positive effect of SDS on performance was confirmed by Enting et al. (2005), who found that birds given diets with pea-corn (SDS) as starch source grew faster and were more feed efficient than those fed a tapioca-corn (RDS) based diet. In addition, this effect was more pronounced when the diet was low in amino acids, again indicating an asynchrony between starch and protein in diets with RDS only.

Gutiérrez del Alamo et al. (2009) tested the effect of wheat starch digestion rate on broiler performance by using different varieties of wheat. Two additional diets were formulated where 25 or 50% of wheat was replaced by pea, slowing starch digestion rate. Although some variation

has been found on the digestion rate of wheat depending on variety or geographical location of growth, wheat is digested more rapidly than pea. It was found that performance was best when chickens were fed the mixture 75% wheat – 25% pea, while the mixture 50% wheat – 50% pea resulted in the poorest performance, with diets using wheat alone resulting in intermediate performance. This suggests that inclusion of a source of SDS at low, but not high, concentrations in broiler chicken diets may improve performance. In addition, they found that these differences were more pronounced in the starter and grower period, but they disappeared afterwards. Additionally, no effect on glycemic index was found in this case.

2.10 Conclusions

The nature of the starch sources can greatly affect digestibility of starch and the physiological response of the bird. Evidence shows that the inclusion of sources that are more slowly digested can have a positive impact on broiler performance. However little is known on the effect of starch digestion characteristics on slow growing chickens such as laying hens or the mechanisms involved both at the physiological and behavioural levels.

2.11 Objectives

The primary objective was to determine the effects of starch digestibility rate and extent on the performance, physiology and behaviour of broiler chickens and laying hens using semi-purified starch sources.

In order to achieve this, specific objectives were:

- To determine the effect of starch digestibility characteristics on the performance of broiler chickens and laying hens using semi-purified starch sources.
- To determine the effect of starch digestibility on broiler digestive tract emptying and content pH under feed withdrawal conditions.
- To confirm the increased presence of starch in the distal small intestine as dietary pea starch increases.

- To evaluate the effect of rate and extent of starch digestion on physiological and morphological parameters of the digestive tract associated with the activation of the ileal brake in broilers and laying hens.
- To assess the effect of slowly or poorly digestive starch inclusion in chicken diets on feeding behaviour of broilers and laying hens.

2.12 Hypotheses

Slowly or poorly digested starch inclusion will improve chicken production efficiency, and this effect will be the result of the activation of the ileal brake due to the increased presence of starch along the digestive tract. More specifically:

- The inclusion of slowly or poorly digestible starch in broiler and laying hen diets will result in a more feed efficient broiler and laying hen production.
- Under feed withdrawal conditions, inclusion of slowly or poorly digested starch will result in a longer presence of nutrients and lower pH values in the digestive tract over the feed withdrawal period.
- Undigested starch in the digesta of small intestine sections will increase as dietary pea starch increases.
- Activation of the ileal brake will result in increased digesta content in the crop, proventriculus and gizzard, higher level of carbohydrate fermentation as reflected by lower pH and higher short chain fatty acid production, as well as increased GLP-1 and PYY ileal gene expression and blood levels.
- Activation of the ileal brake will result in increased digesta retention time.
- Slowly or poorly digested starch will increase satiety as reflected by longer time between feeding bouts.

3.0 Assessing the effect of rate and extent of starch digestion on broiler chicken performance

The objective of this chapter was to confirm the effects of slowly digestive starch on broiler performance, but with the use of semi-purified sources. In addition to previous research focusing on growth and feed efficiency, Chapter 3 evaluates further effects of pea starch percentage by assessing meat yield and digestive tract emptying under feed withdrawal conditions.

3.1 ABSTRACT

Dietary starch with a lower rate and extent of digestion improves broiler feed efficiency, but previous results might have been confounded by non-starch components of the grains. Therefore, the objective of this research was to study the effects of starch digestion on broilers using semi-purified starch sources. Semi-purified wheat (WS, rapidly digested) and pea (PS, slowly digested) starch were combined to create six WS:PS ratios (100:0, 80:20, 60:40, 40:60, 20:80, 0:100) in starter, grower and finisher diets. Each treatment was fed to Ross 308 male (2,124) and female (2,376) broilers housed in 72 litter floor pens from 0-31 d of age to measure performance and meat yield relative to live weight. The effect of diet on feed withdrawal was assessed on 20 males per treatment on d 33. Data were analyzed with ANOVA and linear and quadratic regression analyses using SAS 9.4. Significance was accepted at $P \leq 0.050$. Body weight gain declined linearly with increasing PS. Feed intake decreased with increasing PS for males, but PS did not affect female feed intake. Mortality corrected gain:feed ratio was quadratically influenced by diet (estimated maximum at 25% PS). Breast meat increased linearly with PS concentration, while fat pad, and breast and thigh skin decreased linearly. Quadratic responses were found for thigh meat and whole drum (estimated maximum values at 56 and 54% PS, respectively). Males grew faster, ate more and had higher mortality than females. They also had larger pectoralis major, thigh bone and whole drum, while females had larger pectoralis minor and more breast and thigh skin. After feed withdrawal, digesta content decreased linearly with time in all sections, except for the crop and duodenum, which showed a quadratic decline. Ileum digesta pH increased linearly with time, while crop and caeca pH reacted quadratically, decreasing before steadily increasing. Diet did not affect digestive tract emptying or digesta pH. In conclusion, dietary PS improved feed efficiency and breast meat yield but did not affect digesta clearance after feed withdrawal.

3.2 INTRODUCTION

Poultry flocks have a high-energy requirement, which they derive from dietary starch, lipid and protein. In the majority of poultry diets, starch in cereal grains is the most important energy source, and for which poultry have a high digestive capacity (Svihus, 2014). However,

feed ingredients vary in starch characteristics that affect its rate and extent of digestion. Among the factors affecting starch digestion are granule size, amylose:amylopectin ratio, and the degree of encapsulation and crystallinity (Parada and Aguilera, 2011). For humans, starch has been classified according to its *in vitro* digestion rate (Englyst et al., 1992). According to this procedure, starch can be classified as rapidly (RDS) or slowly digested (SDS) depending on how long it takes for digestion to occur. Starch can also be classified as resistant (RS) when digestion does not occur within a pre-determined period of time. These terms reflect consequences of the rate and extent of starch digestion *in vivo* (Lee et al., 2011b). Among the most prominent of these consequences are effects on post-prandial metabolism and fermentation of resistant starch (e.g. Seal et al., 2003; Alviña and Araya, 2004; Bolhuis et al., 2010; Souza da Silva et al., 2014), 2014). Similar terms can also be used in animal feeding, but the exact classification definitions may require modification to match the digestive tract of the animal.

Differences in the rate of starch digestion can have important consequences. These include post-prandial metabolism, bacterial fermentation, and direct and indirect effects on the small intestine (SI). For instance, post-absorptive effects, including a reduction in glycemic index, occur when SDS is consumed in contrast to RDS in meal-fed animals (Meynier et al., 2015). SDS could also provide energy to distal SI enterocytes, potentially sparing the use of amino acids for this purpose (Enting et al., 2005). Additionally, it is possible that SDS activates nutrient sensing mechanisms related to unabsorbed nutrients such as the ileal brake, thereby enhancing the extent of digestion by increasing transit time, among other aspects of digestive tract function (Shin et al., 2013b). Starch presence in more distal SI increases the potential for fermentation due to the increased microbial load in distal rather than proximal areas of the SI (Yeoman et al., 2012). Undigested starch can similarly be fermented in the SI, caeca and colon. Preferential fermentation by microbes and resulting fermentation products (short-chain fatty acids) have the potential to modify microbial populations along the digestive tract (Regmi et al., 2011), thereby affecting bird health and resistance to colonization by zoonotic organisms. In summary, utilization of SDS results in gradual glucose absorption that may more closely match an animal's physiological energy requirements, allowing the immediate utilization of glucose for processes such as muscle deposition instead of energy storage (Deep et al., 2018). Also, SDS fermentation products may enhance intestine barrier function, thereby reducing the need for immune response activation.

The inclusion of SDS sources in poultry diets has been shown to have a positive effect on performance (Weurding et al., 2003; Gutiérrez del Alamo et al., 2009). Weurding et al. (2003) selected five lysine concentrations and two starch sources, tapioca-corn (TC) and pea-corn (PC), after confirming by *in vitro* assay that TC was more rapidly digested than PC. Their results indicate that regardless of the amount of lysine in the diet, PC diets resulted in an average 2% improvement in feed efficiency when fed to broiler chickens. In another study, five isoenergetic and isonitrogenous diets with different starch digestion rates were fed to broiler chickens. The authors found that broiler growth and performance responded in a quadratic fashion, with an improvement of 1.34% in FCR when the starch digestion rate was decreased by 14.3% (Gutiérrez del Alamo et al., 2009). However, an even lower digestion rate proved to be detrimental.

Another effect of feeding SDS could be on digestive tract emptying after feed withdrawal prior to slaughter. Based on activation of the ileal brake, SDS could slow digestive passage with either negative or positive consequences. If too slow, there could be increased potential for contamination at slaughter. In contrast, a more prolonged digestion period and delayed glucose absorption time might spare the depletion of glycogen stores during transportation to processing, affecting body weight loss and meat quality (Pethick et al., 1995). A longer presence of feed in the digestive tract may also enhance fermentation in the crop and distal digestive tract, thereby decreasing pH via short-chain fatty acids production, and reducing the Salmonella colonization associated with increased pH (Hinton et al., 2000; Byrd et al., 2001).

Although research supports the benefits of formulating poultry diets that incorporate SDS, it is possible that other components of the grain starch sources used in previous research confound the interpretation of results. Provided that starch digestibility characteristics are maintained, the use of semi-purified starch sources may avoid this problem. Thus, the objectives of this study were to determine the effects of starch digestibility characteristics on [1] the performance of broiler chickens using semi-purified starch sources, and [2] digestive tract emptying and content pH under feed withdrawal conditions. The hypotheses are that [1] the inclusion of slowly or poorly digestive starch in broiler diets will result in a more feed efficient broiler production, and that, [2] under feed withdrawal conditions, inclusion of slowly or poorly

digested starch will result in a more extended presence of nutrients and lower pH values in the digestive tract over the feed withdrawal period.

3.3 MATERIALS AND METHODS

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (Canadian Council on Animal Care, 1993, 2009).

3.3.1 *Experimental treatments*

To study the effect of starch digestion rate and extent on broiler performance, six diets were formulated to be identical (Table 3.1), with the exception of the starch fraction composition, which contained graded levels of semi-purified wheat (WS) and pea starch (PS) ranging from 100% WS to 100% PS. Semi-purified WS (Archer Daniels Midland Company, Montreal, QC, Canada) and PS (Parrheim Foods, Saskatoon, SK, Canada) were chosen after confirming differences in starch digestion characteristics via *in vitro* analysis. In accordance with previous research using intact wheat and pea (Weurding et al., 2001), PS was found to be digested more slowly than WS. Because the purity of the starch sources was not equal (WS = 92.5% *vs*. PS = 80.0% starch), purified pea protein (Parrheim Foods, Saskatoon, SK, Canada) was added to the WS to equalize differences in starch, protein and fibre concentrations. The starch fraction of the 0% PS diets contained 87% WS and 13% pea protein. Thereafter, PS replaced a percentage of the WS-pea protein mixture (20, 40, 60, 80 or 100%) to make the rest of the treatments. Diets were formulated to meet or exceed nutrient requirements for broiler chickens (Aviagen, 2014). The feed was manufactured at the Canadian Feed Research Centre (CFRC, North Battleford, SK, Canada). Due to the fine characteristics of the ingredients, diets were pelleted with the conditioning temperature not exceeding 85°C.

3.3.2 *Birds and bird housing*

A total of 4,500 Ross x Ross 308 broiler chicks were obtained from a commercial hatchery (Sofina Inc., Wynyard, SK, Canada) and allocated randomly by gender to 12 pens in each of six rooms at the Poultry Centre at the University of Saskatchewan (Saskatoon, Canada).

Table 3.1. Ingredient composition and nutrient content of treatment diets

Ingredient (%)	Starter	Grower	Finisher
Semi-purified starch	47.49	53.66	59.35
Soybean meal	39.08	32.65	27.31
Porcine meal	5.00	5.00	5.00
Oat hulls	2.00	2.00	2.00
Soybean oil	2.71	3.50	3.36
Mono-calcium phosphate	0.97	0.78	0.68
Limestone	1.19	0.92	0.86
Sodium chloride	0.37	0.37	0.37
Vitamin/mineral premix[1]	0.50	0.50	0.50
Choline chloride	0.10	0.10	0.10
DL-Methionine	0.52	0.46	0.42
L-Threonine	0.07	0.06	0.05
Calculated nutrient composition (%)			
AME (kcal/kg)	3,025	3,150	3,200
Dry matter	88.61	87.99	88.22
Crude protein	25.09	22.43	20.24
Crude fat	4.34	5.14	5.01
Calcium	1.05	0.90	0.85
Available phosphorus	0.50	0.45	0.42

AME: apparent metabolizable energy.
Starter (0.4 kg/bird) and grower (1.4 kg per birds) were fed based on chick placement; the finisher diet was fed from then until the end of the trial.
[1]Supplied per kilogram of diet: vitamin A (retinyl acetate + retinyl palmitate), 11000 IU; vitamin D_3, 2200 IU; vitamin E (dl-α-tocopheryl acetate), 300 IU; menadione, 2.0 mg; thiamine, 1.5 mg; riboflavin, 6.0 mg; niacin, 60 mg; pyridoxine, 4 mg; vitamin B_{12}, 0.02 mg; pantothenic acid, 10 mg; folic acid, 0.6 mg; biotin, 0.15 mg; copper, 10 mg; iron, 80 mg; manganese, 80 mg; iodine, 0.8 mg; zinc, 80 mg; selenium, 0.3 mg; calcium carbonate, 500 mg; antioxidant, 0.625 mg; wheat midds, 3772.73 mg.

The experiment was a randomized complete block design with 12 diet by gender treatments in each room; the room was assigned as the blocking unit.

Birds were raised under controlled conditions of light (23L:1D, 30 lux during the first 2 d followed by 16L:8D, 10 lux until the end of the experiment) and temperature (starting at 33°C and reduced daily to reach 21°C by d 25). Feed and water were offered *ad libitum*. Wheat straw was used as bedding material in floor pens (4.6 m^2). Each pen was furnished with a tube feeder

(36 cm pan diameter from 0-21 d and 43 cm diameter after that) and six nipple drinkers. Based on 32 d weights obtained from the Ross Performance objectives (Aviagen, 2014), 66 female or 59 male chicks were allocated to each pen, to achieve a final estimated bird density of 31 kg/m^2.

3.3.3 *Chemical analyses*

Before diet formulation, digestibility of purified starch sources was assessed *in vitro* according to the technique described by Karunaratne et al. (2018). Briefly, 700 mg of the starch source (0.5 mm) and 50 mg of guar gum powder were measured in triplicate. After adding 1.5 ml of enzyme solution I (2000 U/ml pepsin-HCl solution) to the tubes and vortexing, they were incubated for 30 min in a 41°C water bath. Following incubation, 20 ml of sodium acetate buffer and 5 ml of enzyme solution II (pancreatin, 28.5 U/ml of amyloglucosidase and 60 U/ml of invertase) were added to each tube and returned to the shaking water bath (41°C, 35 mm stroke length, 160 strokes/min). Aliquots from each tube were collected at 15, 30, 45, 60, 90, 120, 180 and 240 min and the reaction was stopped immediately by adding 50 ml of polypropylene to each sample. Glucose was measured colourimetrically (D-Glucose Assay procedure – GOPOD format, K-GLUC 07/11, Megazyme Inc., Chicago, IL, USA) after centrifuging each tube at 1500 rpm for 2 min to obtain the supernatant.

Diets were analyzed for total starch (TS), moisture, fat, crude protein (CP), ash, and dietary fibre (DF; Table 3.1) following standard AOAC methods (AOAC, 2006). Starch was determined using the Megazyme Total Starch Kit (Megazyme Inc., Chicago, IL, USA) following the 996.11 AOAC method. Moisture was measured using method No. 930.15. Fat content was determined by ethyl ether extraction using Goldfish Extraction Apparatus (Labconco model 35001; Labconco, Kansas, MO, USA) following AOAC method 920.39. Crude protein of the diets was determined using a Leco N analyzer (Leco FP-528; Leco Corp., St. Joseph, MI, USA) following method 990.03 and a 6.25 multiplication factor was used to convert N to CP. Ash was measured (method 942.05) using a muffle oven (Lindberg/Blue BF51842, Asheville, NC 28804, USA). Dietary fibre was determined using Megazyme Total Dietary Fiber kit (Megazyme Inc., Chicago, IL) following the AOAC 985.29 method.

3.3.4 *Data collection*

Body weight and feed intake were measured on a pen basis at 0, 6, 10, 17, 24 and 31 d of age. Mortality was considered in feed intake and feed efficiency (gain:feed) calculations. Mortality and culls were collected, weighed, recorded daily, and sent to be necropsied at Prairie Diagnostic Services (Saskatoon, SK, Canada). On d 31, 18 birds per treatment (3 birds per pen) were randomly selected for meat yield determination and sent to the slaughter plant. The returned carcasses were cut into: breast (pectoralis major and minor, skin), left drum (skin, meat, bone), left thigh (skin, meat, bone), intact right drum, intact right thigh, wings, abdominal fat and back rack. All sections were weighed and presented as a percentage of live weight.

On d 31, another 20 males per treatment from four pens (5 males/pen) were randomly selected for a feed withdrawal study and kept under the same housing and management conditions until the start of the study 48 h later. On d 33, feed was removed 5 h after dawn to avoid crop involvement as a result of increased feed intake during the early morning, followed by water removal and crating 4 h later, to mimic birds feeding and drinking status prior to and during transportation to a slaughter plant. Simultaneously with feed removal, and 2, 4, 6 and 8 h later, four birds per treatment were weighed and euthanized by intravenous injection with T61 Euthanasia solution (0.35 ml/kg body weight; Merck & Co., Inc. Kirkland, QC, Canada). Digestive tracts were removed, sectioned from crop to colon, and full and empty weights recorded. *In situ* pH was assessed in crop, middle ileum and right caecal contents (pH Meter model Phi 34, Beckman Instruments, Inc.; Fullerton, CA, USA).

3.3.5 *Statistical analyses*

Normality of the residuals and homogeneity of variance was checked in all collected data previous to statistical analysis. Data was transformed when required to meet the assumptions. *In vitro* starch analysis data were analyzed at each time point using a one-way ANOVA (MIXED model procedure). Performance data were analyzed in a randomized complete block design, using room as a blocking factor, diet and gender as the main effects, and pen as the experimental unit. Room effects were not significant ($P > 0.05$) and therefore room was removed from analyses and data were analyzed as a completely randomized design. Growth and production data were analyzed using ANOVA (MIXED model procedure), and regression analyses (Proc

reg, linear relationships; Proc Rsreg, quadratic relationships) to determine the effect of the relative amount of PS present in the diet with SAS statistical software (version 9.4, SAS Institute, 2004). Arcsin square root or logarithm transformation were applied when necessary to meet statistical assumptions. Feed withdrawal data were analyzed with ANOVA (MIXED model procedure) and regression analyses (Proc reg; Proc Rsreg) for each of the main effects (time and PS concentration). Square root or logarithm transformations were applied when necessary. All differences were considered at $P \leq 0.05$ and trends were considered when $0.10 \geq P > 0.05$.

3.4 RESULTS

The *in vitro* digestibility assay demonstrated that semi-purified WS was digested more rapidly and extensively than semi-purified PS. After 15 min, the digestibility values were 45% for WS and 21% of PS. By 90 min most of WS was digested (94%), while only 88% of PS was hydrolyzed after 120 min of digestion (Figure 3.1). Starch digestibility between WS and PS differed significantly at all time points with the exception of time = 0 min *($P \leq 0.05$)*.

Formulation of the diets was based on laboratory analysis of samples of the ingredients provided by Archer Daniels Midland Company (Montreal, QC, Canada) and Parrheim Foods (Saskatoon, SK, Canada). However, batch differences between the analyzed semi-purified PS sample and the one used to make the diets resulted in a gradual reduction of the starch content of the diets as the percentage of PS increased; other diet components gradually increased to compensate for the reduced starch.

Chick initial body weight was 40±0.0001 g, and did not differ between treatments. Body weight gain from 0 to 28 d of age decreased as the amount of PS in the diets increased, from 1.90 to 1.77 kg, with a trend to being quadratic ($P = 0.096$) reaching an estimated maximum at 22% PS (Tables 3.2 and 3.3). Males gained 200 g more than females by 31 d of age. An interaction between the main factors showed that diet did not affect feed intake for females, which on average consumed 2.64 kg of feed per bird, but feed intake decreased linearly from 2.98 to 2.85 kg per bird for males as the concentration of PS increased. A quadratic dietary effect on gain:feed corrected for mortality was seen for the 0-31 d period, with an estimated maximum at 25% PS; females were less feed efficient than males. Average mortality was 4.15%, and no

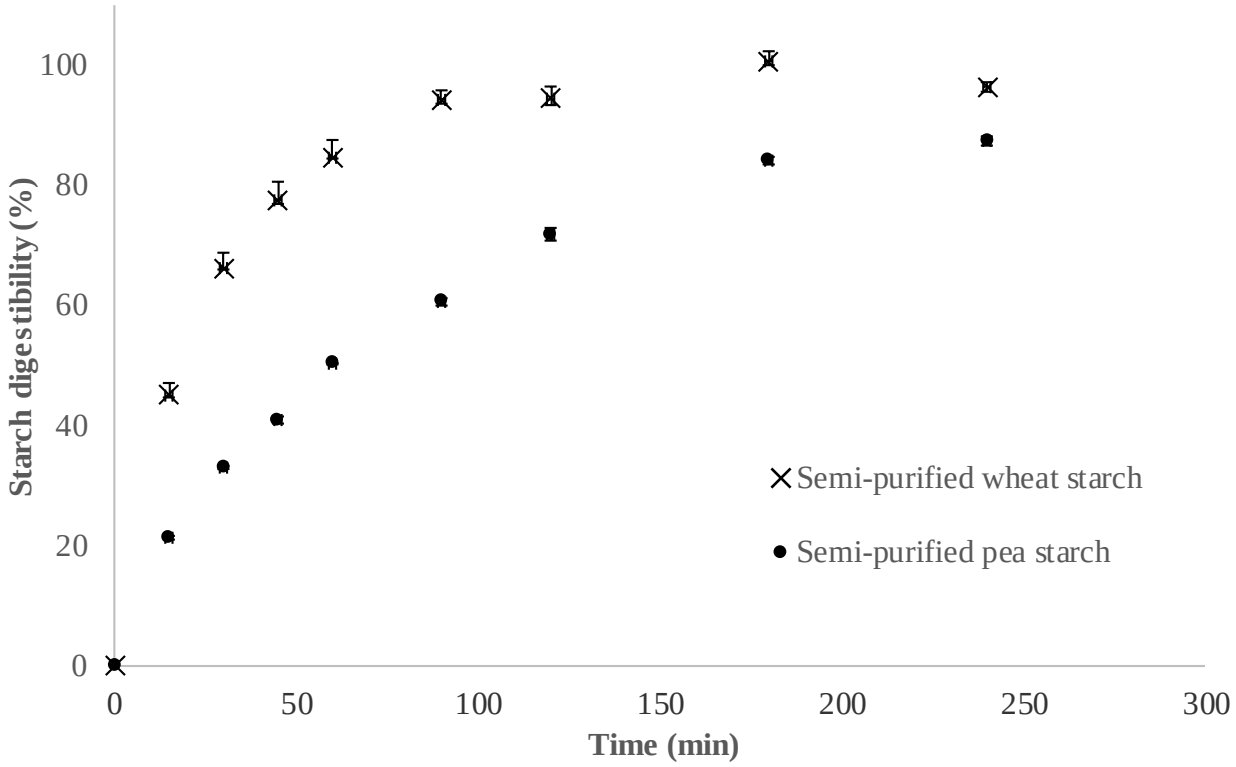

Figure 3.1. Percentage of starch disappearance through *in vitro* analysis of semi-purified wheat and pea starch at 0, 15, 30, 45, 60, 90, 120, 180 and 240 minutes of the small intestine phase. Samples were analyzed in triplicate. Each data point represents the mean value +/- standard deviation. ANOVA analysis resulted in statistical differences at all time points ($P \leq 0.05$) with the exception of time = 0 min.

Table 3.2. Effect of the proportion of dietary wheat and pea starch and gender on growth, feed intake, feed efficiency and mortality of broiler chickens from 0 to 31 d of age

	Diet (D)							Gender (G)			DxG	SEM
	0[1]	20	40	60	80	100	*P* value	M	F	*P* value		
BWG (kg)	1.90	1.93	1.91	1.87	1.85	1.77	<0.001	1.97	1.77	<0.001	NS	0.015
FI (kg/bird)	2.79	2.79	2.80	2.76	2.74	2.79	NS	2.91	2.64	<0.001	0.036	0.018
G:F_m	0.69	0.70	0.69	0.68	0.68	0.64	<0.001	0.68	0.68	0.050	NS	0.003
Mort. (%)	4.06	4.66	4.56	4.25	3.69	3.65	NS	4.85	3.44	0.028	NS	0.310

[1] Diet denominations indicate the percentage of pea starch in the starch fraction of the diet.
P value: indicates ANOVA *P* value; M: Male; F: Female; SEM: Pooled standard error of the mean; BWG: body weight gain; FI: feed intake; G:F_m: gain:feed corrected for mortality; Mort: mortality percentage (includes culls); number of replications = 6 pens.

Table 3.3. Relationship between pea starch concentration and growth, feed intake, feed efficiency and mortality of broiler chickens from 0 to 31 d of age

	Regression	R^2	Equation
BWG (kg)	L=0.002	0.13	$y = -0.001x + 1.94$
Male FI (kg/bird)	L=0.010	0.18	$y = -8_x10^{-4}x + 2.96$
Female FI (kg/bird)	NS	-	-
G:F_m	Q<0.001	0.49	$y = -3_x10^{-5}x^2 + 0.001x + 0.69$
Mort. (%)	NS	-	-

L: linear regression *P* value; Q: quadratic regression *P* value; BWG: body weight gain; FI: feed intake; G:F_m: gain:feed corrected for mortality; Mort: mortality percentage (includes culls); number of replications = 6 pens.

differences were found between dietary treatments; mortality was higher in males than females (4.80 *vs*. 3.45%). Likewise, diet did not affect the cause of mortality. Most broilers died from infectious and metabolic causes (1.58 and 0.89% respectively). The remaining birds died due to skeletal (0.40%) or other causes (0.42%) with a 0.84% where the mortality cause was unknown.

Total breast meat weight increased linearly with PS from 19.3 to 20.3% of body weight, while thigh meat and whole drum increased in a quadratic fashion reaching an estimated maximum at 56 and 54% PS, respectively (Tables 3.4 and 3.5). Abdominal fat, and breast and drum skin decreased linearly with increasing PS (0.81 to 0.65, 3.12 to 2.80 and 0.48 to 0.44 % of body weight, respectively). Males had larger relative weights for pectoralis major muscle, whole drum, drum meat, and thigh and drum bones, and smaller weights for pectoralis minor muscle, breast skin, and thigh skin than females. No gender or treatment effects were found for carcass, whole thigh or wing weights. An interaction between diet and gender was found for the carcass portion (back and rack) remaining after meat and limb removal. Female back and rack changed quadratically with an estimated maximum at 40% PS. Male back and rack showed a trend to turn quadratically ($P = 0.058$) with an estimated minimum at 46% PS. No other differences were found.

Under feed withdrawal conditions, no interactions were found between diet and the number of hours off feed for digestive tract content, and digesta pH. Concentration of PS did not affect digesta pH, or digestive tract content weights (Table 3.6). The crop (quadratic), ileum (linear) and caeca (quadratic) digesta pH increased with increasing time off feed, reaching a pH of 6.00, 7.14 and 6.94 respectively after 8 h of feed withdrawal (Table 3.7). Digestive tract content decreased with time off feed in a linear (gizzard, jejunum, ileum, caeca and colon) or quadratic (crop, duodenum) fashion. The crop was completely empty between 2 and 4 h after feed withdrawal started, while jejunum and ileum were close to empty after 8 h.

3.5 DISCUSSION AND CONCLUSIONS

Plant source affects the physicochemical characteristics in starch granules (Weurding et al., 2001; Zhang et al., 2006; Gutierrez del Alamo et al., 2009; Singh et al., 2010; Lee et al., 2011). However, less is known on how these differences impact chicken digestibility

Table 3.4. Effect of the proportion of dietary wheat and pea starch and gender on meat yield of broiler chickens (31 d)

	Diet (D)							Gender (G)			DxG	SEM
	0[1]	**20**	**40**	**60**	**80**	**100**	***P* value**	**M**	**F**	***P* value**		
Live weight (kg)	2.06	1.99	2.0	1.98	1.90	1.88	<0.001	2.11	1.83	<0.001	0.006	0.021
Relative weight (% of live weight)												
Carcass	69.9	71.5	71.5	70.8	70.6	70.6	NS	70.7	70.9	NS	NS	0.25
Total breast	19.3	20.2	20.3	20.0	20.4	20.3	NS	20.2	19.9	NS	NS	0.12
Pectoralis major (2)	16.2	16.8	16.8	16.7	17.0	16.9	NS	17.0	16.5	0.022	NS	0.10
Pectoralis minor (2)	3.13	3.43	3.42	3.32	3.37	3.41	0.013	3.27	3.42	0.007	NS	0.029
Breast skin (2)	3.12	3.18	2.89	2.91	2.95	2.80	0.044	2.83	3.12	<0.001	NS	0.043
Abdominal fat pad	0.81	0.75	0.61	0.66	0.59	0.65	NS	0.66	0.69	NS	NS	0.023
Thigh, whole (1)	6.0	6.1	6.1	6.0	6.0	6.0	NS	6.0	6.1	NS	NS	0.03
Thigh meat (1)	4.3	4.4	4.5	4.5	4.4	4.4	NS	4.5	4.4	NS	NS	0.03
Thigh skin (1)	1.1	0.9	0.9	1.0	0.9	0.9	NS	0.8	1.1	<0.001	NS	0.03
Thigh bone (1)	0.75	0.77	0.77	0.77	0.77	0.82	NS	0.80	0.76	0.012	NS	0.008
Drum, whole (1)	4.7	4.7	4.8	4.8	4.8	4.7	NS	4.8	4.7	0.023	NS	0.02
Drum meat (1)	3.0	3.0	3.1	3.1	3.0	3.0	NS	3.1	3.0	0.002	NS	0.02
Drum skin (1)	0.48	0.50	0.49	0.46	0.44	0.43	NS	0.47	0.47	NS	NS	0.008
Drum bone (1)	1.21	1.17	1.17	1.22	1.23	1.20	NS	1.23	1.17	0.008	NS	0.010
Wings (2)	7.4	7.4	7.4	7.4	7.5	7.3	NS	7.4	7.4	NS	NS	0.03
Back and rack	17.2	17.3	17.5	16.8	17.1	17.2	NS	17.1	17.2	NS	0.007	0.09

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.

P value: indicates ANOVA *P* value; M: Male; F: Female; SEM: Pooled standard error of the mean. Numbers in brackets indicate the number of parts included in the measurement; number of replications = 6 pens.

Table 3.5. Relationship between pea starch concentration and meat yield of broiler chickens (31 d)

	Regression	R^2	Equation
Male live weight (kg)	L<0.001	0.45	$y = -0.003x + 2.24$
Female live weight (kg)	NS	-	-
Relative weight (% of live weight)			
Carcass	NS	-	-
Total breast	L=0.037	0.07	$y = 0.007x + 19.72$
Pectoralis major (2)	NS	-	-
Pectoralis minor (2)	NS	-	-
Breast skin (2)	L=0.007	0.09	$y = -0.003x + 3.14$
Abdominal fat pad	L=0.008	0.09	$y = -0.002x + 0.76$
Thigh, whole (1)	NS	-	-
Thigh meat (1)	Q=0.023	0.08	$y = -6_x10^{-5}x^2 + 0.007x + 4.28$
Thigh skin (1)	NS	-	-
Thigh bone (1)	NS	-	-
Drum, whole (1)	Q=0.025	0.07	$y = -5_x10^{-5}x^2 + 0.005x + 4.65$
Drum meat (1)	NS	-	-
Drum skin (1)	L=0.002	0.12	$y = -7_x10^{-4}x + 0.50$
Drum bone (1)	NS	-	-
Wings (2)	NS	-	-
Male back and rack	NS	-	-
Female back and rack	Q=0.023	0.20	$y = -3_x10^{-4}x^2 + 0.02x + 17.10$

L: linear regression *P* value; Q: quadratic regression *P* value; numbers in brackets indicate the number of parts included in the measurement; number of replications = 6 pens.

Table 3.6. Effect of the proportion of dietary wheat and pea starch on digestive tract pH and digesta weights of broiler chickens on up to 8 h of feed withdrawal (33 d)

	Diet						Regression		SEM
	0[1]	**20**	**40**	**60**	**80**	**100**	***P* value**	**R^2**	**Equation**	
BW (kg)	2.34	2.22	2.30	2.18	2.27	2.19	NS	-	-	0.021
pH										
Crop	5.22	5.40	5.61	5.19	5.16	5.28	NS	-	-	0.069
Ileum	6.68	6.87	6.62	6.99	6.55	6.67	NS	-	-	0.071
Caeca	6.80	6.70	6.75	6.75	6.63	6.66	NS	-	-	0.031
Contents (g, as-is basis)										
Crop	3.2	4.3	2.5	3.8	3.0	2.7	NS	-	-	0.70
Proventriculus	0.4	0.7	0.4	0.3	0.9	0.3	NS	-	-	0.31
Gizzard	6.1	6.3	7.3	6.1	7.8	8.7	NS	-	-	0.47
Duodenum	2.2	2.2	2.1	2.3	2.4	2.3	NS	-	-	0.08
Jejunum	8.4	8.9	7.1	9.2	10.7	8.5	NS	-	-	0.60
Ileum	6.9	6.6	6.7	9.1	9.9	8.8	NS	-	-	0.68
Caeca	5.2	5.0	4.8	5.1	5.6	6.3	NS	-	-	0.22
Colon	0.9	0.8	0.7	0.8	0.9	0.9	NS	-	-	0.07

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.
P value: indicates ANOVA *P* value; L: linear regression; Q: quadratic regression; SEM: pooled standard error of the mean. BW: body weight; number of replications = 4 pens.

Table 3.7. Effect of time after feed withdrawal on digestive tract pH and digesta weights of broiler chickens (33 d)

	Time (h)					Regression			SEM
	0	**2**	**4**	**6**	**8**	***P* value**	**R^2**	**Equation**	
BW (kg)	2.25	2.26	2.31	2.27	2.16	NS	-	-	0.021
pH									
Crop	4.99	4.88	5.19	5.50	6.00	Q=0.020	0.27	$y = 0.02\ x^2 - 0.03x + 4.96$	0.069
Ileum	6.63	5.92	7.00	6.97	7.14	L<0.001	0.17	$y = 0.11x + 6.30$	0.071
Caeca	6.66	6.66	6.69	6.63	6.94	Q=0.029	0.09	$y = 0.01\ x^2 - 0.06x + 6.69$	0.031
Contents (g, as-is basis)									
Crop	12.1	2.8	0.6	0.4	0.3	Q<0.001	0.33	$y = 0.36\ x^2 - 4.15x + 11.34$	0.70
Proventriculus	1.0	0.7	0.2	0.3	0.2	L<0.001	0.09	$y = -0.11x + 0.94$	0.31
Gizzard	9.7	8.1	5.7	6.8	5.0	L=0.001	0.12	$y = -0.62x + 9.22$	0.47
Duodenum	2.2	2.5	2.6	2.3	1.8	Q=0.007	0.08	$y = -0.03\ x^2 + 0.22x + 2.18$	0.08
Jejunum	15.5	13.2	7.1	5.6	2.7	L<0.001	0.50	$y = -1.65x + 15.35$	0.60
Ileum	14.8	14.0	7.9	2.4	0.8	L<0.001	0.55	$y = -1.94x + 15.75$	0.68
Caeca	5.4	6.4	5.8	5.1	4.0	L=0.002	0.11	$y = -0.26x + 6.15$	0.22
Colon	1.3	1.1	1.0	0.5	0.3	L<0.001	0.26	$y = -0.13x + 1.33$	0.07

L: linear regression *P* value; Q: quadratic regression *P* value; SEM: Pooled standard error of the mean. BW: body weight; number of replications = 4 pens.

(Weurding et al., 2001; Gutiérrez del Alamo et al., 2009). In addition, components other than starch present in grains (protein, fat, other carbohydrates, anti-nutritional factors, etc.) may confound the effects attributed to the nature of the starch. To eliminate or reduce this potential issue, diets were formulated using semi-purified starch. *In vitro* analysis of WS and PS digestibility validated their use in this study. Addition of pea protein to the WS was an effective way of equalizing differences (quantitatively and qualitatively) between WS and PS other than the starch itself, and analyses of the diets (data not shown), as well as *in vivo* digestibility values (Chapter 5) support its use. However, dietary analyses showed a reduction of the starch concentration, as PS inclusion increased as a result of the PS having a lower than expected starch level. Thus, although we were successful in separating starch from starch source, the reduction in the concentration of starch introduced a confounding effect.

Despite issues with the formulation of our diets, effects of rate and extent of starch digestion were observed. Our results support previously reported findings (Weurding et al., 2003; Gutiérrez del Alamo et al., 2009) that low concentrations of slowly digested starch are beneficial for broiler performance (feed efficiency, weight gain). However, our findings also demonstrate that medium and particularly high concentrations of PS are detrimental. The reduction in dietary starch as PS increased could be partially responsible for these findings, but other factors may also contribute to the reduced performance. *In vitro* digestibility results show that a higher proportion of PS than WS would not be digested in the SI. Similarly, *in vivo* research demonstrated that 3.2% of the starch in the terminal ileum was undigested in birds fed 100% PS in comparison to 0.9% for birds fed the 0% PS diets (Chapter 5). These results are also supported by other *in vivo* comparisons of wheat and pea starch digestibility (Weurding *et al.*, 2001). The lower starch digestibility of high PS diets will either result in lost energy via excreta or fermentation of the starch, with the consequent loss in energy-yielding potential (Wiseman, 2006). No interactions between the main effects were found, except for feed intake, with gender effects following the usual trends (males showing higher feed intake and a faster growth rate than females), indicating no gender differences in the mechanisms involving the dietary effects. Likewise, mortality levels were within the normal range, indicating no negative effects of the diets on bird health, at least in a low infectious disease environment.

Breast meat increased with PS while body fat was reduced. The increase in muscle while body fat is reduced suggests a shift in energy partitioning from fat to muscle as dietary PS increases. This hypothesis is supported by previous findings reported by Deep et al. (2015). In their experiment, either wheat- or pea-based diets were fed to broiler breeder pullets under a skip-a-day feeding schedule (unpublished data). Their data demonstrated that feeding pea reduced both liver weight and fat content, and correspondingly reduced the expression of key lipogenesis enzymes (acetyl-CoA carboxylase, malic enzyme) in comparison to wheat-fed birds. The authors hypothesize that feeding pea (more slowly digested starch) in comparison to wheat (rapidly digested starch) reduced the need for energy storage between meals. Whether that holds true in *ad libitum* fed broilers where birds eat at short intervals (Weeks et al., 2000; Shynkaruk, 2017; Chapter 7) has not been investigated. However, it is of interest that in an experiment run concurrently with the present study and using the same diets, a trend ($P = 0.087$; Chapter 5) was noted for a linear decrease in liver weight with increasing dietary PS concentration, suggesting that similar mechanisms may be occurring in *ad libitum* fed broilers. The above data indicate that starch digestion rate can affect bird metabolism and nutrient partitioning, but more research is required to confirm this finding and also investigate specific metabolic effects.

It was hypothesized that inclusion of PS would increase transit time, increasing the time contents would remain in the digestive tract and in turn increase the potential for fermentation and decreased content pH. No effects of PS concentration on feed withdrawal digestive tract emptying or pH were observed, rejecting the hypothesis. Time effects support previous publications with a total digestive tract emptying 8 h after feed withdrawal started and digestive tract pH levels increasing with time (e.g. Veerkamp, 1986; Corrier et al., 1999; Sengor et al., 2006). An issue during processing of broilers is *Salmonella* contamination, with highest population numbers reported to be in the crop (Hargis et al., 1995; Ramirez et al., 1997). Fermentation by crop *Lactobacilli* minimize the increase in *Salmonella* numbers by competitive exclusion and maintaining a low pH (Byrd et al., 2001). The presence of feed in the crop during the first 2 h after feed withdrawal kept pH low. Once feed was gone, the observed steady increase in pH suggests an opportunity for a growth in *Salmonella* numbers risking carcass contamination. The rapid increase of crop pH after feed disappearance highlights the importance of promoting crop involvement to reduce the chance of *Salmonella* contamination (Byrd et al., 2001).

In conclusion, although the effect of intrinsic characteristics of starch sources have been mostly overlooked in the past, the inclusion of low concentrations of starch sources with lower digestion rate and extent in poultry diets results in beneficial effects on efficiency and meat yield production. Further analysis of the causes, including the expression of enzymes involved in energy partitioning, as well as its application using practical diets is necessary. Meanwhile, no dietary effects on feed withdrawal parameters were found, leading to the acceptance of the second null hypothesis.

4.0 Assessing the effect of rate and extent of starch digestion on laying hen performance

Although the positive effect of PS on broiler performance was confirmed, no research has been published in relationship to slowly digested starch in laying hens. The objective of this chapter was to evaluate the effects of slowly digested starch on laying hens using semi-purified starch sources. Data collection included egg production, egg quality, feed intake, feed efficiency, body weight gain, body weight uniformity and feather cover.

4.1 ABSTRACT

The inclusion of starch with a lower rate and extent of digestion has proven to be beneficial in broiler production. However, less is known about its effect on laying hen performance. Therefore, six diets were formulated to produce differing ratios (0:100, 20:80, 40:60, 60:40, 80:20 and 100:0) of semi-purified wheat (WS; rapidly digested) and pea starch (PS; slowly digested). Each diet was fed to 120 conventionally caged Lohmann LSL lite hens in groups of 12, from 26 to 46 wk of age, and its effects on performance and feather covering were assessed. Data were analyzed by regression analysis, and the significance level was chosen at $P \leq 0.05$. Hen-day egg production (HDP) was high (97.05%) and unaffected by PS concentration for 0-10 weeks of the trial, followed by a quadratic-shaped HDP, with an estimated maximum at 67% PS concentration, for the 10-20 week period of the trial. Overall, a positive effect of PS was found on HDP which increased linearly with PS. No effect on egg weight was found (average weight = 59.6±2.1 g), but eggshells were thickest at the 55% PS concentration. Body weight gain was affected by PS concentration and maximized at 49% PS. Body weight uniformity and mortality were not affected by dietary treatment. Feed intake increased linearly with PS from 102 to 109 g/hen/day, while feed:egg mass ratio was minimal at 26% PS. Using a scale from 1-4 per body part (neck, wings, back, vent and breast), only neck feather cover increased linearly with PS, from 3.0 (0% PS) to 3.2/4 (100% PS). However, back and total feather coverage showed a trend ($P = 0.054$ and $P = 0.079$ respectively) to increase linearly with PS as well (from 3.7 to 3.9/4 and 15.6 to 16.6/20, respectively). In summary, feeding PS at low to intermediate concentrations improved laying hen performance. Further research should focus on the mechanisms involved in this effect.

4.2 INTRODUCTION

Chickens have a limited capacity to digest fat, particularly when they are young (Wiseman et al., 1998), but a high level of secretion of amylolytic enzymes provides them with an impressive starch digestion capacity (Lehrner and Malacinski, 1975; Svihus, 2014). This and the affordability of starch sources has made starch the most important energy source in poultry diets.

The rate and extent of starch digestibility are affected by source (Weurding et al., 2001). Starch sources commonly used in poultry diets, such as wheat and corn, have ileal digestibility coefficients between 94 and 99%, while others, such as pea and beans, reach only 81% digestion. When a portion of traditional starch ingredients in poultry diets is replaced by sources with a lower rate and extent of digestion, broiler chickens respond with an enhanced production efficiency (Weurding et al., 2003; Gutiérrez del Alamo et al., 2009; Chapter 3).

Weurding et al. (2003) found an average improvement of 1.98% in broiler production feed efficiency when feeding diets containing pea-corn instead of tapioca-corn, after confirming that the starch from the latter treatment was more rapidly digested. A similar result was found by Gutierrez del Alamo et al. (2009) when diets, based on differing digestion rates, were fed to broilers. It was found that when starch digestion rate, defined as the proportion of starch digested per h, was reduced from 2.48 to 2.17 h^{-1}, broiler feed efficiency was improved by 1.32%, but even lower digestion rates were detrimental. In both studies, diets were manufactured using starch sources based on the entire grain, with potential confounding effects from other grain components. However, similar results were found when semi-purified starch sources were used instead (Chapter 3). In the latter study, semi-purified wheat starch (WS) and semi-purified pea starch (PS) were chosen as starch sources for the broiler diets. Dietary treatments differed in starch composition from 100% WS to 100% PS, with graded combinations in between. Results showed an improvement of 1.59% in production feed efficiency when a combination of 80% WS and 20% PS was used when compared to 100% WS. In addition, breast meat yield increased as the proportion of PS increased, while breast skin and fat pad decreased, suggesting a metabolic effect of the rate and extent of starch digestion on energy partitioning.

Evidence supports a beneficial effect of using a combination of starch that includes low concentrations of starches with a lower rate and extent of digestion in broiler diets. However, little to no research has been done regarding its effects on laying hens. In order to better understand how laying hens would respond under similar dietary treatments, the objective of this study was to determine the effect of starch digestibility rate and extent on the performance of laying hens using semi-purified starch sources. It was hypothesized that inclusion of PS in laying hen diets would affect nutrient allocation and metabolism, improving egg production and feed efficiency.

4.3 MATERIALS AND METHODS

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (Canadian Council on Animal Care, 1993, 2009).

4.3.1 *Experimental treatments*

To study the effect of starch digestion rate and extent on laying hen performance, six diets were formulated to contain graded levels of semi-purified WS and PS, ranging from 100% WS to 100% PS (Table 4.1) in the starch fraction of the diets. Semi-purified WS (Archer Daniels Midland Company, Montreal, QC, Canada) and PS (Parrheim Foods, Saskatoon, SK, Canada) were chosen after *in vitro* analysis confirmed differences in starch digestion characteristics (Chapter 3). PS was found to be digested more slowly and to a lesser extent than WS, which is supported by previous *in vivo* and *in vitro* research with complete wheat and pea sources (Weurding et al., 2001). Because the purity of the semi-purified starch sources was not equal (WS = 92.5% vs. PS = 80.0%), purified pea protein (Parrheim Foods, Saskatoon, SK, Canada) was added to the WS to overcome differences in fibre and protein content. Diets were formulated to meet or exceed the nutrient requirements of laying hens (Lohmann Management guide, 2016), and were manufactured at the Canadian Feed Research Centre (CFRC, North Battleford, SK, Canada). Due to the fine characteristics of the ingredients, diets were pelleted. The conditioning temperature was maintained under 85°C.

4.3.2 *Birds and bird housing*

A total of 720 d old Lohmann LSL chicks (Clark's Poultry Inc., Brandon, MB, Canada) were litter floor raised at the University of Saskatchewan Poultry Centre under controlled environmental conditions. At 16 weeks of age, pullets were randomly housed, six birds per cage, in 120 Specht conventional laying hen cages (503 cm^2/bird, 60 cm feeder trough, one Lubing nipple drinker). Cages were located in the same tier, and two adjacent cages were considered an experimental unit, resulting in 10 replications of 12 hens per treatment diet (6 diets), arranged in a completely randomized design. The barn was maintained at approximately 21^oC; birds were

Table 4.1. Ingredient composition of treatment diets

	Diets (% pea starch)					
Ingredients (%)	**0**	**20**	**40**	**60**	**80**	**100**
Wheat starch	46.57	37.25	27.94	18.63	6.52	0.00
Pea protein	6.85	5.48	4.11	2.74	0.96	0.00
Pea starch	0.00	10.68	21.37	32.05	45.94	53.42
Soybean meal	26.30	26.30	26.30	26.30	26.30	26.30
Porcine meal	5.00	5.00	5.00	5.00	5.00	5.00
Oat hulls	2.00	2.00	2.00	2.00	2.00	2.00
Soybean oil	1.51	1.51	1.51	1.51	1.51	1.51
Limestone	9.65	9.65	9.65	9.65	9.65	9.65
Mono-calcium phosphate	0.70	0.70	0.70	0.70	0.70	0.70
Sodium chloride	0.34	0.34	0.34	0.34	0.34	0.34
Vitamin/mineral premix[1]	0.50	0.50	0.50	0.50	0.50	0.50
Choline Chloride	0.10	0.10	0.10	0.10	0.10	0.10
DL-Methionine	0.43	0.43	0.43	0.43	0.43	0.43
L-Threonine	0.05	0.05	0.05	0.05	0.05	0.05
Calculated composition (% DM)						
AME (kcal/kg)	2,800	2,800	2,800	2,800	2,800	2,800
Dry matter	89.79	89.79	89.79	89.79	89.79	89.79
Crude protein	19.35	19.35	19.35	19.35	19.35	19.35
Digestible Lys	1.17	1.17	1.17	1.17	1.17	1.17
Digestible Met	0.58	0.58	0.58	0.58	0.58	0.58
Digestible Met+Cys	0.84	0.84	0.84	0.84	0.84	0.84
Digestible Thr	0.74	0.74	0.74	0.74	0.74	0.74
Calcium	4.10	4.10	4.10	4.10	4.10	4.10
Available phosphorus	0.42	0.42	0.42	0.42	0.42	0.42

[1]Supplied per kilogram of diet: vitamin A (retinyl acetate + retinyl palmitate), 8000 IU; vitamin D_3, 3000 IU; vitamin E (dl-α-tocopheryl acetate), 25 IU; menadione, 1.5 mg; thiamine, 1.5 mg; riboflavin, 5.0 mg; niacin, 30 mg; pyridoxine, 1.5 mg; vitamin B_{12}, 0.012 mg; pantothenic acid, 8.0 mg; folic acid, 0.5 mg; biotin, 0.06 mg; copper, 5 mg; iron, 80 mg; manganese, 80 mg; iodine, 0.8 mg; zinc, 80 mg; selenium, 0.3 mg; calcium carbonate, 500 mg; ethoxyquin, 0.625 mg; wheat middlings, 3822.79 mg.

provided with 14 h of light per d (10 lux), and feed and water on an *ad libitum* basis. The trial started when hens were 26 weeks of age and continued for 20 weeks.

4.3.3 *Data collection*

The total number of eggs per experimental unit and the presence of cracks, broken, double, soft or abnormal eggs were recorded daily from Monday to Friday during the length of

the experiment, to be later mathematically corrected to seven days a week. Hen-day (HDP) and hen-housed production (HHP) were calculated as the total number of eggs per time period per experimental unit divided by the number of hen-days and hens housed, respectively. Starting at 28 weeks of age and thereafter every four weeks, all eggs laid per experimental unit over a period of 24 h were collected, individually weighed, and eggshell thickness was assessed using specific gravity (Wells, 1966). Egg mass was calculated by multiplying the total number of eggs by the average egg weight.

Individual body weights were measured at the beginning and the end of the experiment and body weight gain and uniformity were calculated. Uniformity was estimated by determining the percentage of hens falling within 10 or 15% of the average body weight per experimental unit. Feed intake was measured per experimental unit every four weeks and feed efficiency was calculated as g of feed per g of egg mass. Daily starch intake was calculated by multiplying daily feed intake by the proportion of starch present in each diet according to chemical analysis. Mortality was collected, weighed and recorded daily. Cause of death was determined by pathologists at Prairie Diagnostic Services (Saskatoon, SK, Canada).

At the end of the experiment, feather coverage of every bird was scored in five body parts: neck, wings, back, vent and breast, by two trained scorers. The scoring system ranged from 1 (less than 25% of feather covering) to 4 (more than 75% of feather covering) for each body part following the procedure reported by Davami et al. (1987). Score data were averaged between scorers before data analyses. A total score per bird was calculated by adding the average score of the five body parts assessed.

4.3.4 *Chemical analyses*

Prior to diet formulation, digestibility of purified WS and PS was assessed *in vitro* following a modified version of Englyst et al. (1992) that approximates chicken digestion (Chapter 3).

Diets were analyzed for starch (TS), moisture, fat, crude protein (CP), ash, minerals and dietary fibre (DF) according to AOAC (2006) procedures. Starch was determined using the Megazyme Total Starch Kit (Megazyme Inc., Chicago, IL, USA) following method 996.11. Moisture was measured following method No. 930.15. Fat content was determined by ethyl ether

extraction using Goldfish Extraction Apparatus (Labconco model 35001; Labconco, Kansas, MO, USA) following AOAC method 920.39. The nitrogen content of diets was determined using a Leco N analyzer (Leco FP-528; Leco Corp., St. Joseph, MI, USA; method 990.03) and derived values were subsequently multiplied by 6.25 to convert to CP. Ash was measured (method 942.05) using a muffle oven (Lindberg/Blue BF51842, Asheville, NC 28804, USA). Mineral analysis of the diets was performed by SGS Agrifood Laboratories (Guelph, ON, Canada) following methods A202, A203a and A204a. Dietary fibre was determined using Megazyme Total Dietary Fiber kit (Megazyme Inc., Chicago, IL) following the AOAC 985.29 method, which determines total dietary fibre by measuring soluble and insoluble fibre fractions.

4.3.5 *Statistical analyses*

Normality of the residuals and homogeneity of variance was checked in all collected data previous to statistical analysis. Data was transformed when required to meet the assumptions. Regression analyses (Proc reg for linear regression; Proc Rsreg for quadratic regression analysis) were performed with SAS (version 9.4, SAS Institute, 2004) using diet as the main factor. Quadratic critical values were obtained from the Proc Rsreg procedure. Data were normality checked, and logarithm+1 transformed when required to comply with statistical assumptions. All differences were considered at $P \leq 0.05$ and trends were considered when $0.10 \geq P > 0.05$.

4.4 RESULTS

For the first ten weeks of the experiment (27 to 37 weeks of age), egg production (HD and HH) was high (Lohmann Tierzucht, 2016) and not affected by the nature of dietary starch (Table 4.2). However, for the second ten-week period (37 to 47 weeks of age), HD egg production responded in a quadratic fashion to increasing PS with the estimated highest production occurring at the 67% PS concentration. Hen-housed egg production during this period tended ($P = 0.069$) to increase linearly with increasing PS. Overall (27 to 47 weeks of age), HD egg production increased linearly with PS, with a trend ($P = 0.052$) to respond quadratically with an estimated maximum at 70% PS, which approximated the production level shown in the Lohmann Management Guide (average HD egg production = 95.64 %; Lohmann Tierzucht, 2016). Overall HH production was not affected by dietary starch composition. Dietary treatment

Table 4.2. Effect of the proportion of dietary wheat and pea starch on the egg production and egg quality of Lohmann LSL lite hens (27-47 weeks of age)

	Diets						Regression		SEM
	0[1]	**20**	**40**	**60**	**80**	**100**	***P* value**	**R^2**	**Equation**	
27-37 weeks of age										
HDP (%)	97.04	96.03	97.23	97.67	97.43	96.91	NS	-	-	0.211
HHP (%)	96.47	95.67	95.10	95.85	96.85	96.00	NS	-	-	0.341
37-47 weeks of age										
HDP (%)	89.42	91.42	93.72	94.13	94.58	92.94	Q=0.008	0.24	$y = -0.001x^2 + 0.15x + 89.22$	0.047
HHP (%)	85.68	88.90	87.15	91.43	91.57	89.02	NS	-	-	0.777
Overall (27-47 weeks of age)										
HDP (%)	93.30	93.76	95.52	95.40	96.03	94.95	L=0.012	0.10	$y = 0.02x + 93.76$	0.294
HHP (%)	91.08	92.28	91.13	96.64	94.21	92.51	NS	-	-	0.521
Egg weight (g)	59.5	59.5	59.9	59.7	59.6	59.6	NS	-	-	0.13
Egg mass	1515	1535	1526	1565	1572	1542	L=0.033	0.02	$y = 0.4x + 1522$	18.3
Specific gravity	1.084	1.085	1.086	1.086	1.086	1.084	Q<0.001	0.05	$y = 1_x10^{-6}x^2 + 1_x10^{-4}x + 1.0833$	0.0003

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.
HDP: hen-day production. HHP: hen-housed production; Egg mass: average kg of egg per hen per 4 wk period; L: linear regression; Q: quadratic regression; SEM: pooled standard error of the mean; number of replications = 10 pairs of cages.

also did not affect the overall number of double, soft, cracked, broken or abnormal eggs. The average egg weight was lower than reported by Lohmann, 59.6±2.1 *vs*. 60.8 g (Lohmann Tierzucht, 2016), and was not affected by treatment. Specific gravity responded quadratically to the concentration of PS, reaching an estimated maximum at 55% PS.

No significant difference in initial hen body weight was found, and body weight remained within breeder guidelines during the study (Lohmann Tierzucht, 2016; Table 4.3). Similarly, although uniformity decreased considerably during the length of the trial, particularly in extreme diets (0% and 100% PS, 20% and 26% change respectively), no effect of diet was found on uniformity. Average uniformity was 69% (10% of the mean) and 86% (15% of the mean) at the end of the experiment. A trend ($P = 0.069$) for a quadratic effect for 10% uniformity was found at the end of the experiment however, reaching an estimated maximum at 53% PS. Body weight gain changed quadratically with PS, with an estimated maximum at 49% PS.

Daily feed consumption increased linearly with PS concentration from 102 to 109 g/hen (Table 4.3), while daily starch consumption followed a quadratic shape response with an estimated minimum at 46.4% PS inclusion. Feed efficiency (feed to egg mass ratio) changed quadratically with PS, with an estimated minimum at 26% PS. The average mortality was 7.0% with no differences in overall mortality or mortality causes among treatments. Most hens died from cage layer fatigue (3.9%), while metabolic, infectious and other causes each reached 0.6% mortality. The remaining 1.4% showed no visible cause of death.

Feather cover tended ($P = 0.079$) to increase linearly with PS from 15.6 to 16.6 out of a maximum score of 20 (Table 4.4). Pea starch had a positive linear effect on neck feather coverage, while back feather coverage similarly showed a trend to increase with PS ($P = 0.054$).

4.5 DISCUSSION AND CONCLUSIONS

Diets in this study were formulated to contain equal amounts of starch. However, a lower starch content of the semi-purified PS used to make the diets when compared to the samples provided for formulation purposes, resulted in a gradual reduction of the total starch as PS inclusion increased. Although this introduced a confounding effect on the observed results, the confounding effect originating from the gradual reduction of the total dietary starch should have

Table 4.3. Effect of the proportion of dietary wheat and pea starch on body weight, uniformity, body weight gain, feed intake, starch intake, efficiency and mortality of Lohmann LSL lite hens (27-47 weeks of age)

	Diets							Regression	SEM
	0[1]	**20**	**40**	**60**	**80**	**100**	***P* value**	**R^2**	**Equation**	
Initial (27 weeks of age)										
BW (kg)	1.62	1.60	1.60	1.61	1.61	1.62	NS	-	-	0.004
Uniformity (10%)	83	83	89	84	83	87	NS	-	-	1.2
Uniformity (15%)	98	96	98	98	95	96	NS	-	-	0.7
Final (47 weeks of age)										
BW (kg)	1.68	1.66	1.71	1.74	1.70	1.63	Q<0.001	0.02	$y = -3_x10^{-5}x^2 + 0.003x + 1.65$	0.009
Uniformity (10%)	63	69	66	76	76	61	NS	-	-	2.2
Uniformity (15%)	86	82	87	89	91	83	NS	-	-	1.5
Overall (27-47 weeks of age)										
Body weight gain (g)	51	55	105	126	88	16	Q<0.001	0.30	$y = -0.03x^2 + 3x + 34$	7.8
Daily feed intake (g)	102	104	105	106	109	109	L<0.001	0.43	$y = 0.07x + 102$	0.5
Daily starch intake (g)	50.4	47.4	49.4	45.9	46.5	46.6	Q=0.041	0.40	$y = 5_x10^{-4}x^2 - 0.08x + 50.1$	0.27
Efficiency (g feed/g egg)	1.85	1.87	1.84	1.86	1.91	1.94	Q=0.002	0.11	$y = 2_x10^{-5}x^2 - 9_x10^{-4}x + 1.86$	0.005
Mortality (%)	8.3	7.5	9.2	5.0	4.2	7.5	NS	-	-	1.05

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.

HDP: hen-day production. HHP: hen-housed production; L: linear regression; Q: quadratic regression; SEM: pooled standard error of the mean; number of replications = 10 pairs of cages.

Table 4.4. Effect of the proportion of dietary wheat and pea starch on the feather cover of Lohmann LSL lite hens (47 weeks of age)

	Diets						Regression		SEM
	0[1]	**20**	**40**	**60**	**80**	**100**	***P* value**	**R^2**	**Equation**	
Neck	3.0	2.9	2.9	3.1	3.0	3.2	L=0.042	0.07	y = 0.003x + 2.9	0.05
Wings	3.5	3.3	3.3	3.5	3.4	3.6	NS	-	-	0.04
Back	3.7	3.6	3.8	3.9	3.9	3.9	NS	-	-	0.04
Vent	2.9	2.9	3.1	3.2	2.9	3.2	NS	-	-	0.06
Breast	2.5	2.4	2.5	2.5	2.5	2.7	NS	-	-	0.04
Total	15.6	15.0	15.5	16.2	15.7	16.6	NS	-	-	0.21

Scoring system per body part: 1 <25% feather coverage; 2 = 25-50% coverage; 3 = 50-75% coverage; 4 >75% coverage.

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.

L: linear regression; Q: quadratic regression; SEM: pooled standard error of the mean; number of replications = 10 pairs of cages.

been linear. In contrast, quadratic effects were found in egg production, body weight gain, eggshell quality and the digestive tract morphology among other parameters, suggesting minimal effects due to the total starch reduction in the diets.

Hen-day egg production increased linearly with PS. However, a trend for a quadratic response predicted that 70% PS inclusion maximized production. This result, as well as the heavier body weights in middle ranges of PS inclusion, suggest that when a combination of starches with differing digestion rate and extent is used, more nutrients can be allocated to growth and production, without the emergence of adverse effects. Like HD egg production, feed intake increased with PS, resulting in a linear daily increase in protein intake from 18.4 g, in 0% PS diets, to 20.8 g, in hens consuming 100% PS diets, which could explain the increase in egg production (Gleaves et al., 1977). However, Kumar et al. (2018) demonstrated that HD egg production increases with digestible lysine (D-Lys) intake (balanced amino acids) up to 769 mg/h/d. Any value above this level did not produce differences in egg production. In the current trial, all hens consumed at least 1,193 mg of D-Lys daily, discarding the protein intake argument as a potential explanation. In fact, no effect of PS on total egg weight was observed either, and it is well documented that the balanced D-Lys intake to maximize egg weight is higher than for egg production (Kumar et al., 2018).

Alternatively, post-prandial metabolic changes associated with starch digestion may also have affected hen performance. Deep (2018) fed broiler breeder pullets wheat- (rapidly digested starch) or pea- (slowly digested starch) based diets and found that starch type affected hepatic metabolism by altering the gene expression levels of a number of enzymes, including acetyl-CoA carboxylase, malic enzyme and apolipoprotein-II. Feeding a pea-based diet also positively affected physiological and behavioural indicators of satiety. Thus, it is possible that the nature of dietary starch produces a long-term metabolic effect that depends on the type of starch and its level of inclusion in poultry diets. In the current study, the finding that egg production was not affected by dietary treatment for the first 10 weeks of the trial, but it was affected in a quadratic fashion for the last 10 weeks (reduced performance at low and high concentrations of PS; estimated maximum 70% PS) are indicative of a longer-term metabolic effect. Dietary PS also resulted in a quadratic shaped body weight gain response (range 16 to 126 g/hen; maximum 60% PS). Although the weight gain differences were small and the nature of the gain (protein or fat)

was not established, this appears to be a metabolic effect that is unrelated to nutrient intake. It is possible that nutrient partitioning in favour of increased production is a component of a metabolic effect. In research with broiler chickens fed diets similar to the current study, an increase in dietary PS concentration decreased fat-pad and skin weight while increasing breast muscle deposition (Chapter 3), suggesting a partitioning of nutrients towards protein synthesis and away from fat deposition. Supportive of a shift away from fat deposition in the current work is the finding that hen relative liver weight decreased with increasing dietary PS (Chapter 6). Insulin is a major regulator of glucose metabolism and therefore a key hormone response to starch digestion rate (Boden et al., 1991; Chida et al., 2000); as a consequence, it is potentially responsible for metabolic changes associated with feeding pea starch. In addition to its essential role in glucose metabolism, it also plays a key role in protein synthesis (Proud, 2006; Tesseraud et al., 2007). Overall, the data suggest that feeding PS modifies hen metabolism and may contribute to differences in production characteristics.

No effect of PS on egg weight was observed. Dietary PS concentration did affect the specific gravity of the eggs, with the response curve predicting that eggshells would be thickest at 55% PS inclusion. Although diets included 2% oat hulls to promote gizzard development, tissue collection showed a small average gizzard size (11 g or 0.63% of body weight; Chapter 6). Nevertheless, an effect of starch type on gizzard size and crop content was found, with larger gizzard sizes and more digesta contents found in the middle ranges of PS concentration (Chapter 6). These findings suggest longer retention of digesta in the gizzard, thereby potentially improving calcium solubilization (Guinotte et al., 1995; Svihus, 2011).

Feed consumption increased linearly with PS during the length of the experiment. Among the hypotheses proposed to explain the positive performance effects of starch that is digested more slowly or to a lesser extent, such as PS, is the activation of the ileal brake. This is a nutrient sensing mechanism by which enteroendocrine L-cells secrete glucagon-like peptide 1 (GLP-1) and peptide tyrosine-tyrosine (PYY). Activation of L-cells occurs in response to fatty acids, carbohydrates, and short-chain fatty acids derived from fermentation of starch present in the luminal contents of the distal jejunum and ileum (Shin et al., 2013a). Metabolic effects of these neuropeptides include a decreased rate of gastric emptying and gastric motility, a modulation of gastrointestinal secretions and an increase in satiety (van Avesaat et al., 2015). Although this

mechanism has been studied mostly in mammals, it has been demonstrated that chickens have GLP-1 and PYY containing L-cell that have similar effects in poultry (Honda et al., 2017), including a reduction in feed intake. Dietary inclusion of starch sources with a lower rate and extent of digestion should increase the amount of starch available for digestion and fermentation in the distal small intestine, increasing L-cell activation. Evidence of this was obtained in Chapter 5, where birds fed the 100% PS diet had 70% (proximal jejunum) to 256% (distal ileum) more luminal starch than hens fed the 0% PS diet. However, the increase in laying hen feed intake with increasing dietary PS concentration does not support the activation of the ileal brake hypothesis. Body weight gain, uniformity and specific gravity values show that intermediate combinations of WS and PS produced better results than the extreme diets. Since egg production increased linearly with the concentration of PS, a higher nutrient demand could have required a higher daily feed intake. At the same time, the higher egg production could also be the result of increased feed intake. It is also likely that diet AME decreased with increasing PS because of the lower levels of starch and starch digestibility as PS concentration increased. As a result of this, a least some of the increase in feed intake with PS could be compensating for a possible reduction of dietary AME (Classen, 2017).

Interestingly, although the effect of PS on body weight and feed intake are different from those previously reported in broilers, the highest production efficiency was obtained at a similar PS inclusion level (laying hen = 26% PS *vs*. broiler = 25% PS; Chapter 3). This result is in accordance with previous findings in broilers when comparing the effects of starches with differing starch digestion characteristics (Weurding et al., 2003; Gutierrez del Alamo et al., 2009; Chapter 3), and suggests that the observed increase in egg production cannot be solely explained by feed intake. As mentioned above, elements of the ileal brake mechanism activation could be involved. Evidence in mammals has shown that a positive correlation has been found between ovulation and increased concentrations of GLP-1 and PYY (Gosman et al., 2006). GLP-1 and PYY have shown to promote LH and FSH release, increasing the allocation of resources towards egg production.

Alternative mechanisms to the ileal brake have been proposed. Weurding et al. (2003) and Enting et al. (2005) found that the positive effect of feeding slowly digested starch increased when diets were lower in amino acids. It was suggested that the use of starch with a lower rate

and extent of digestion in poultry diets, which has a lower glycemic index, could improve the synchronization between starch and protein digestion, thereby improving performance. However, this argument requires confirmation since, under *ad libitum* feeding, nutrients are continuously being digested and absorbed throughout the digestive tract of chickens. Additional mechanisms include the sparing of amino acid oxidation in the distal small intestine enterocytes by the presence of starch (Weurding et al., 2003), and starch fermentation which could have prebiotic benefits, releasing short-chain fatty acids and improving barrier function (Woodward et al., 2012).

Total feather cover showed a trend to increase linearly with PS, driven mostly by changes in neck and back coverage. In a cage environment, feather coverage can be poor due to the damage caused by contact with the cage, particularly in the neck area as a result of feeding events. However, in this case, neck coverage showed the opposite effect to that expected according to feed intake, and an analysis of feeding behaviour showed no effect of PS concentration on the number of visits to the feeder, frequency or total feeding time of laying hens (Chapter 7). While other body parts such as breast feather cover can be negatively affected by the cage wear, through sitting or dustbathing, changes in the feather cover of the back area are likely due to feather pecking (Bilcik and Keeling, 1999). Hence, the positive effect of PS on neck feather cover, in addition to the strong trend observed in the back area, suggest that PS reduced feather pecking behaviour. In another study where wheat- or a pea-based feed were fed to broiler breeders pullets, differences in the behavioural repertoire were found (Deep, 2018). Birds in pea-based diet showed more object pecking (including feather pecking) and comfort behaviour, while there was a decrease in drinking, standing, and foraging. Although the effects observed in behaviour differ between trials, it is further evidence that feeding pea can affect bird metabolism and subsequently behaviour. Alternatively, microbial metabolism has been found to correlate with feather pecking behaviour in hens as well (Meyer et al., 2013). When comparing high and low feather pecking birds, low feather pecking was correlated with higher protein (ammonia, putrescine, cadaverine) and lower carbohydrate fermentation (short chain fatty acids) in the caeca. While PS inclusion allowed for more starch fermentation in the distal ileum of broilers fed similar diets to the current experiment, with the net production of lactate, no significant amounts of starch reached the caeca (Chapter 5). As starch disappeared along the small intestine, microbial fermentation in the caeca should shift towards other digesta components such as

protein, thereby reducing feather pecking and improving feather cover according to the results presented by Meyer et al. (2013). However, no indications of increased protein fermentation were found in the caeca of laying hens with the increasing PS concentration (no change in pH between treatments; Chapter 6). Therefore, it seems unlikely, that the PS concentration effect observed on feather pecking could be due to protein fermentation.

In conclusion, incorporation of PS in laying hen diets can have positive effects on production performance at low to intermediate concentrations by improving egg production, feed efficiency, egg-shell quality and feather cover.

5.0 Assessing the effect of rate and extent of starch digestion on ileal brake activation in broiler chickens

After confirming the positive effect of PS on broiler performance, the involvement of the ileal brake as one of the potential participating mechanisms was assessed. The objective of the following chapter was to confirm the increased presence of starch along the digestive tract when PS was included in the diets. In addition, the research studied the presence of potential activators (starch, pH, SCFA) and evidence of ileal brake or L-cell activation (changes in the digestive tract, serum and gene expression levels of GLP-1 and PYY). This portion of the research was run concurrently with the experiment presented in Chapter 3, although different rooms were used.

5.1 ABSTRACT

Starch digestion characteristics are known to affect physiological and production parameters in animals. Among the potential effects that arise from differences in starch digestion is activation of the ileal brake. The objective of this research was to evaluate activation of the ileal brake in broiler chickens using diets containing graded levels of semi-purified wheat (WS; rapidly digested) and pea (PS; slowly digested) starch. Starter, grower and finisher diets were formulated to contain six WS:PS ratios (100:0, 80:20, 60:40, 40:60, 20:80, 0:100) and each starch ratio was fed to 236 Ross 308 male broilers housed in 4 litter floor pens. At 28 d of age, the effect of PS concentration was assessed on starch digestion, digestive tract morphology, digesta pH and short-chain fatty acid (SCFA) concentration. Glucagon-like peptide-1 (GLP-1) and peptide tyrosine-tyrosine (PYY) concentrations and expression were assessed through ELISA in serum and jejunum and ileum gene expression (proglucagon for GLP-1). Data were analyzed with linear and quadratic regression analyses. Significance was accepted at $P \leq 0.05$. Starch digestibility decreased linearly or quadratically with increasing diet PS, but digestibility in the colon was not affected by treatment. Crop content pH changed in a quadratic fashion with an estimated minimum at 55% PS, while distal ileum and caecal pH only showed a trend ($P = 0.064$) to change quadratically. Total SCFA increased linearly in the crop, while they changed in a quadratic fashion in the ileum (estimated maximum at 62% PS). Concentration of SCFA in caeca was highest for the 80 and 100% PS concentrations. The relative empty weights of the crop, small intestine and colon increased linearly with increasing PS concentration. Increasing diet PS increased the length of the small intestine (linear). Relative digesta content weight increased with PS concentration in the crop, jejunum and ileum, with no effects in other digestive tract sections. Dietary treatment did not affect serum GLP-1 or PYY or small intestine transcript abundance. In conclusion, feeding PS increased the presence of potential L-cell activators (starch, SCFA) and caused increased trophic development and digesta content of the digestive tract, suggestive of L-cell activation. However, failure of PS to affect GLP-1 and PYY serum concentrations or gene expression would indicate a different L-cell response in broiler chickens or an alternate mechanism is responsible for digestive tract changes.

5.2 INTRODUCTION

In its simplest definition, starch is a polymer of glucose molecules linked by 1-4 and 1-6 α-glycosidic bonds (Asp, 1996). However, its physicochemical characteristics such as the size of the granules, the degree of crystallinity, amylose-amylopectin ratio or presence of other compounds, which depend on plant source, can affect its rate and extent of digestion (Parada and Aguilera, 2011). These differences have resulted in a classification method for starch, which is based on its rate of *in vitro* digestibility. Rapidly digested (RDS), slowly digested (SDS) and resistant (RS) starch classifications are based on digestion times of under 20 min, 20-240 min and over 4 h, respectively, using the *in vitro* procedure described by Englyst et al. (1992). Although developed to mimic starch digestion in mammals, the general concept can also be applied to other monogastric animals including chickens.

Traditional starch sources included in poultry diets (wheat, corn) are rapidly digested and also highly digestible. However, a number of studies have shown that the incorporation of starch with a lower rate and extent of digestion in poultry diets can be beneficial, particularly in terms of feed efficiency (Weurding et al., 2003; Enting et al., 2005; Gutierrez del Alamo et al., 2009; Chapters 3 and 4). Some theories have arisen to explain this effect. Weurding et al. (2001) proposed that slowly digested starch results in synchronization of glucose and amino acid uptake, along with a lower but steadier rise in blood glucose and insulin concentrations, which permit direct utilization of nutrients instead of the less efficient storage of excess glucose. Additionally, when starch is more slowly digested, glucose can be absorbed in the ileum and directly utilized by enterocytes as a readily available energy source, potentially sparing amino acids use for that purpose (Watford et al., 1979). The starch that remains in the digestive tract for a more extended period of time can also be fermented and act as a prebiotic by increasing short-chain fatty acid (SCFA) production, reducing digestive tract pH and affecting the gut bacterial community (Macfarlane and Macfarlane, 2003).

Another mechanism which has not been explored in chickens, and that could affect production efficiency, is the ileal brake. Undigested nutrients in the distal small intestine, particularly fats and carbohydrates, result in the activation of enteroendocrine L-cells, which stimulate the release of glucagon-like peptide 1 (GLP-1; Wachters-Hagedoorn et al., 2006; Tolhurst et al., 2009; Mansour et al., 2013) and peptide tyrosine-tyrosine (PYY; Chan et al.,

2006; Brooks et al., 2017). These peptides are proposed to reduce gastric emptying, which in turn increases digestibility and inhibits feed intake by enhancing satiety (Meyer et al., 1998a; van Avesaat et al., 2015). However, conclusive proof of these mechanisms acting in chickens, particularly the effect on satiety, is still lacking.

In contrast to mammals, both GLP-1- and PYY-immunoreactive cells in chickens are located mainly in the jejunum and ileum, with just a few in the duodenum and none in other sections of the digestive tract (Hiramatsu et al., 2005; Aoki et al., 2017a). Not much research has been done on the type of nutrients that activate GLP-1 or PYY release in chickens. Monir et al., (2014a) found a direct relationship between GLP-1 containing cells and dietary protein; while in another study, contrary to what has been found in mammals, the frequency of GLP-1 immunoreactive cells occurrence was inversely related to feed intake, increasing under low feed intake (Monir et al., 2014b). On the other hand, PYY appears to act in a similar way to that reported in mammals, with increased gene expression under *ad libitum* feeding when compared to fasting (Aoki et al., 2017a). Feed intake was reduced in day-old broiler and laying hen chicks administered with a central injection of GLP-1 (Furuse et al., 1997; Honda et al., 2014), while peripheral injection of PYY in 9 day-old broilers had the same effect (Aoki et al., 2017b). Thus, many of the mammalian ileal brake components have been confirmed in chickens, but some differences warrant further research.

To understand the impact of starch digestion rate and extent on digestive tract morphology and physiology, diets were formulated to contain variable proportions of semi-purified wheat (WS; rapidly digested) and pea (PS; slowly digested) starch and fed to broiler chickens from 0 to 28 d of age. More specifically, the research objective was to evaluate the effect of starch digestion on activation of the ileal brake. It was hypothesized that the presence of starch along the digestive tract would increase with concentration of dietary PS. In addition, starch digestion and fermentation products would be sensed by enteroendocrine L-cells, resulting in the ileal brake mechanism activation. The presence of starch and carbohydrate fermentation (as reflected by pH and SCFA concentration) were measured to assess L-cell activation potential, while digestive tract morphology, digesta content, serum concentrations of GLP-1 and PYY and small intestine abundance of proglucagon (GLP-1 precursor) and PYY transcripts were measured to determine if activation had occurred.

5.3 MATERIALS AND METHODS

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (Canadian Council on Animal Care, 1993, 2009).

5.3.1 *Experimental treatments*

To study the effect of starch digestion rate and extent in the activation of the ileal brake, six diets were formulated to be identical (Table 5.1), with the exception of the source of the starch fraction, which contained graded levels of semi-purified WS and PS ranging from 100% WS to 100% PS. Semi-purified WS (Archer Daniels Midland Company, Montreal, QC, Canada) and PS (Parrheim Foods, Saskatoon, SK, Canada) were chosen after confirming differences in starch digestion rate between sources via *in vitro* analysis (Chapter 3). In accordance with previous research using intact wheat and pea (Weurding et al., 2001), PS was found to be digested more slowly than WS. Because the purity of the starch sources was not equal (WS = 92.5% *vs*. PS = 80.0% starch), purified pea protein (Parrheim Foods, Saskatoon, SK, Canada) was added to the WS to equalize starch, protein and fibre concentrations. The starch fraction of the 0% PS diets contained 87% WS and 13% pea protein. Thereafter, the appropriate amount of this mixture (20, 40, 60, 80 or 100%) was replaced by PS to produce the rest of the treatments. Diets were formulated to meet or exceed the nutrient requirements for broiler chickens (Aviagen, 2014). The feed was manufactured at the Canadian Feed Research Centre (CFRC, North Battleford, SK, Canada). Diets were pelleted with conditioning temperatures not exceeding 85°C; starter diets were crumbled prior to feeding. The finisher diet fed from d 24-28 contained 0.03% TiO_2 as an undigested marker to measure starch digestibility, added on top of formulation.

5.3.2 *Birds and bird housing*

A total of 1,416 Ross x Ross 308 male broiler chicks were obtained from a commercial hatchery (Sofina Inc., Wynyard, SK, Canada) and housed in two independent rooms at the Poultry Centre at the University of Saskatchewan (Saskatoon, SK, Canada). Chicks were allocated randomly to six dietary treatments with four replications each.

Birds were raised under environmentally controlled conditions of light (23L:1D, 18 lux for 2 d followed by 16L:8D at 10 lux until the end of the experiment) and temperature (starting at

Table 5.1. Ingredient composition and nutrient content of treatment diets

Ingredient (%)	Starter	Grower	Finisher
Semi-purified starch	47.49	53.66	59.35
Soybean meal	39.08	32.65	27.31
Porcine meal	5.00	5.00	5.00
Oat hulls	2.00	2.00	2.00
Soybean oil	2.71	3.50	3.36
Monocalcium phosphate	0.97	0.78	0.68
Limestone	1.19	0.92	0.86
Sodium chloride	0.37	0.37	0.37
Vitamin/mineral premix[1]	0.50	0.50	0.50
Choline Chloride	0.10	0.10	0.10
DL-Methionine	0.52	0.46	0.42
L-Threonine	0.07	0.06	0.05
Calculated nutrient composition (%)			
AME (kcal/kg)	3,025	3,150	3,200
Dry matter	88.61	87.99	88.22
Crude protein	25.09	22.43	20.24
Crude fat	4.34	5.14	5.01
Calcium	1.05	0.90	0.85
Available phosphorus	0.50	0.45	0.42

AME: apparent metabolizable energy.
Starter (0.4 kg/bird) and grower (1.4 kg per birds) were fed based on chick placement; the finisher diet was fed from then until the end of the trial.
[1]Supplied per kilogram of diet: vitamin A (retinyl acetate + retinyl palmitate), 11000 IU; vitamin D3, 2200 IU; vitamin E (dl-α-tocopheryl acetate), 300 IU; menadione, 2.0 mg; thiamine, 1.5 mg; riboflavin, 6.0 mg; niacin, 60 mg; pyridoxine, 4 mg; vitamin B12, 0.02 mg; pantothenic acid, 10.0 mg; folic acid, 0.6 mg; biotin, 0.15 mg; copper, 10 mg; iron, 80 mg; manganese, 80 mg; iodine, 0.8 mg; zinc, 80 mg; selenium, 0.3 mg; calcium carbonate, 500 mg; antioxidant, 0.625 mg; wheat midds, 3772.73 mg.

33°C was reduced daily to reach 21°C by d 25). Feed and water were offered *ad libitum*. Wheat straw was used as bedding material in floor pens (4.6 m^2). Each pen was furnished with a tube feeder (36 cm diameter from 0-21 d and 43 cm diameter after) and six nipple drinkers. Groups of 59 chicks were allocated to each pen.

5.3.3 *Data collection*

At 28 d of age, four h after lights came on, seven random birds per pen were individually weighed and euthanized via intravenous injection of T61 Euthanasia solution (0.35ml/kg body weight; Merck & Co., Inc. Kirkland, QC). The digestive tracts were removed from four birds per replication. *In situ* pH was assessed in the crop, ileum and caecal contents using a pH Meter (model Phi 34, Beckman Instruments, Inc.; Fullerton, CA, USA). Each digestive tract was sectioned from crop to colon, and the jejunum and ileum were partitioned into proximal and distal portions. Full and empty weights of each section were recorded, along with the length of small intestine, caeca and colon sections; weights were obtained for the heart, liver and pancreas. All contents, except the proventriculus and gizzard, were collected, pooled per pen and frozen immediately for starch and titanium determination.

Prior to euthanasia, peripheral blood (brachial vein) was collected from the three remaining birds, for determination of serum concentrations of GLP-1 and PYY using ELISA. Samples were allowed to clot and centrifuged for 15 min at 1000 x g. Resulting sera were stored (-20ºC) until further analyses. Following euthanasia and digestive tract removal, digesta content from the crop, distal ileum and caeca were collected into 15 mL centrifuge tubes, sealed immediately and frozen (-20 ºC) for SCFA analysis. Middle jejunum and middle ileum sections (1 cm long) were also collected, immediately frozen in individual bags using liquid nitrogen, and stored at -80ºC for PYY and proglucagon gene expression analyses.

5.3.4 *Sample analyses*

Dietary analysis. Diets were analyzed for starch (TS), moisture, fat, crude protein (CP), ash, dietary fibre (DF) and TiO_2 following AOAC standard methods (2006). Starch was determined using the Megazyme Total Starch Kit (Megazyme Inc., Chicago, IL, USA) following method 996.11. Moisture was measured following method No. 930.15. Fat content was determined by ethyl ether extraction using Goldfish Extraction Apparatus (Labconco model 35001; Labconco, Kansas, MO, USA) following method 920.39. Nitrogen was determined using a Leco N analyzer (Leco FP-528; Leco Corp., St. Joseph, MI, USA; method No 990.03) and the value multiplied by 6.25 to obtain CP. Ash was measured (method 942.05) using a muffle oven (Lindberg/Blue BF51842, Asheville, NC 28804, USA). Dietary fibre was calculated by the

addition of the soluble and insoluble fractions, which were determined using Megazyme Total Dietary Fiber kit (Megazyme Inc., Chicago, IL) following method 985.29. Titanium was determined using the procedure described by Myers et al. (2004). Briefly, 0.5 g of sample was digested at 420°C for 2 h by adding a CT-37 Kjeldahl tablet (3.5 g of K_2SO_4 + 0.4 g of $CuSO_4$; Fisher Scientific, Geel, Belgium) and 13 mL of H_2SO_4. After allowing the samples to cool down for 30 min, 10 mL of 30% H_2O_2 were added, and further cooling was allowed. Next, and after adding distilled water, the precipitate was removed by using filtration paper (Whatman 541, GE Healthcare, Buckinghamshire, UK). Titanium concentration was determined by measuring absorbance at 410 nm and comparing the value to the standard curve built with known titanium concentrations. All dietary measurements were assessed in duplicate except for starch which was measured in triplicate.

Starch digestibility. *In vivo* starch digestibility was evaluated by measuring starch, titanium and dry matter in the proximal and distal jejunum and ileum, and colon digesta contents. Starch, dry matter and titanium were determined as previously described.

Short-chain fatty acid analysis. Determination of SCFA was performed following a modified version of the procedure described by Zhao et al. (2006) measuring the samples in triplicate. Briefly, for SCFA analysis, an internal standard was prepared by diluting 0.5 g of 3-methyl-n-valeric acid in 1 L of 0.15 mol/L of oxalic acid. The standard solution was made by weighing 400 mM of acetic, propionic and butyric acid, 200 mM of isovaleric, valeric and lactic acid, 50 mM of caproic acid and 100 nM of isobutyric acid. After thawing, samples of digesta were weighed in triplicate and then 25% phosphoric acid was added 1:1, mixed and centrifuged (12,500 x g for 5 min). One mL of supernatant in triplicate was collected and placed into microcentrifuge tubes. Internal standard was added at 1:1 ratio to supernatant and centrifuged (12,500 x g for 10 min). Samples were filtered using a 3 mL syringe and a 0.45 µm Nylon filter into a glass GC vial. Concentrations of SCFA were measured using a Thermo Scientific Gas Chromatograph (model Trace 1310, Milan Italy) and a Zebron capillary GC column (ZB-FFAP, length: 30 m, I.D: 0.25 mm, film thickness: 0.25 µm, Phenomenex, Torrance, CA).

Determination of GLP-1 and PYY serum concentrations. The concentration of GLP-1 in serum was measured using Chicken GLP-1 ELISA kit (Catalogue No: E-EL-Ch0160; Elabscience Biotechnology, Wuhan, China). PYY serum concentrations were determined using

Chicken Peptide YY ELISA kit (Catalog No: CSB-EL019128CH, Cusabio Biotechnology, Wuhan, China).

Gene expression. To prepare samples for gene expression analyses through quantitative polymerase chain reaction (qPCR), tissue samples collected from jejunum and ileum were first individually homogenized using mortar and pestle while still frozen under liquid nitrogen. RNA extraction of the samples was performed using TRIzol reagent Ambion (Invitrogen, Carlsbad, CA). Brielfly, 1 mL of TRIzol reagent was added to 60-70 mg of frozen ground tissue and continuously shaken for 5 min. Next, 200 µL of chloroform (Fisher Scientific, Hampton, NH) were added, shaken for 3 min and centrifuged at 12,000 x g at 4°C for 15 min. The aqueous phase was collected, and 500 µL of 100% isopropanol (Acros Organics, Thermo Fisher Scientific, NJ) were added, incubated for 10 min at room temperature, and centrifuged at 12,000 x g at 4°C for 10 min. After discarding the supernatant, the pellet was washed with 1 mL of 75% ethanol and centrifuged at 7,500 x g at 4°C for 5 min. Ethanol was removed, and samples were air-dried for 10 min. Pellets were re-suspended in 20-50 µL of Ambion Nuclease-free water (ThermoFisher Scientific) and incubated at 55-60°C for 12 min.

The concentration and purity of RNA were measured using Nanodrop ND-1000 Spectrophotometer (ThermoFisher). Each sample was diluted accordingly to 500-1500 ng by adding Nuclease-free water. To prepare cDNA a High-Capacity Reverse Transcription kit (Applied Biosystems; Thermofisher Scientific, Foster City, CA) was used. A master mix was prepared with 10X RT Buffer, 25X dNTP mix, 10X RT random primers and MultiScribe reverse transcriptase following the kit instructions and added at a volume of 5.8 µL to each sample. The reaction was performed at 25°C for 10 min, after which temperature was increased to 37°C for 2 h, and followed by 5 min at 85°C using a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The obtained cDNA was stored at -20°C until qPCR reactions were run soon after.

Gene expression was measured using qPCR method. A master mix was made by using 10 µL of SsoFast Evagreen Supermix (Bio-Rad Laboratories, Hercules, CA), 0.8 µL of forward primer (10 µM) and 0.8 µL of reverse primer (10 µM) specific for chicken proglucagon, proglucagon B, PYY, GAPDH or RPS7 (Table 5.2; Invitrogen, Carlsbad, CA), and 6.4 µL of Nuclease-free water per well. Two µL of cDNA and 18 µL of master mix were added to qPCR plates in

Table 5.2. Primers used for quantitative polymerase chain reaction

Gene	Direction	Sequence	Reference
Proglucagon	Forward	5'-CACAAGGCACATTCACCAGT-3'	Richards and McMurtry, 2008
	Reverse	5'-TTCTTTGGCAGCTTGACCTT-3'	
Proglucagon B	Forward	5'-CACAAGGCACATTCACCAGT-3'	Richards and McMurtry, 2008
	Reverse	5'-TGGTATTCTCCCAAAAGGTCTC-3'	
PYY	Forward	5'-AGGAGATCGCGCAGTACTTCT-3'	Aoki et al., 2017b
	Reverse	5'-TGCTGCGCTTCCCATACC-3'	
GAPDH	Forward	5'-GTGAAAGTCGGAGTCAACGGA-3'	Cheled-Shoval et al., 2011
	Reverse	5'-AAGGGATCATTGATGGCCAC-3'	
RPS7	Forward	5'-TAGGTGGTGGCAGGAAAGC-3'	Olias et al., 2014
	Reverse	5'-TTGGCTTGGGCAGAATCC-3'	

duplicate. A no reverse transcriptase control and a no template control were included in duplicate in each plate, as well as a standard curve, which was run in triplicate. Standard curves were assembled by using a concentration of 10 ng of cDNA per microliter for the first standard, followed by 5-fold dilutions. Once covered, each plate was centrifuged, checked for bubbles and set in the CFX Connect Optics Module Real-Time System (Bio-Rad Laboratories, Hercules, CA). Cycling conditions for all genes were identical including denaturation at 95.0°C for 5 min, and annealing and extension as 60.0°C for 5 min. Amplification efficiency for each gene ranged from 86 to 137 % and R^2 values for standard curves ranged from 94.5 to 99.7. Obtained values of proglucagon, proglucagon B and PYY were normalized by the average of GAPDH and RPS7, previous to conducting statistical analyses. Expression of reference genes was not affected by treatment.

5.3.5 *Statistical analyses*

Normality of the residuals and homogeneity of variance was checked in all collected data previous to statistical analysis. Data was transformed when required to meet the assumptions. Regression analyses (Proc reg, linear regression; Proc Rsreg, quadratic regression) were performed with SAS 9.4 (SAS Institute, 2004). Data were checked for compliance with statistical analysis assumptions. No transformation was required for digestibility data. Due to differences in body size, all digestive tract data were divided by body weight to yield proportional values. Logarithm transformations were applied to the digestive tract, ELISA and SCFA data when required to normalize the residuals. Similarly, the square root of gene expression data was used for statistical analysis. All differences were considered at $P \leq 0.05$ and trends were considered when $0.10 \geq P > 0.05$.

5.4 RESULTS

Performance criteria from a simultaneous trial (Chapter 3) show that broilers met or exceeded Aviagen performance criteria (Aviagen, 2014). Diets were formulated based on the analysis of starch samples. However, the starch concentration of the PS used for feed manufacturing was lower than expected (61 vs. 80%). As a result, a gradual reduction of starch content occurred as PS level increased (e.g. 100% PS finisher diets had 6% less starch than its

0% PS counterpart). Nevertheless, analyzed diet nutrients were very similar to the calculated values presented in Table 5.1. Diets were formulated to keep fat and DF concentrations similar to avoid the possibility of ileal brake activation due to components other than starch in the diets (Zhou et al., 2008; van Avesaat et al., 2015). While the resulting fat concentrations were similar among diets (4.9±0.2%), DF concentrations increased with higher concentrations of PS (e.g. finisher DF concentration increased by from 13.9% in 0%PS to 17.4% in 100% PS). However, the soluble fibre fraction accounted for 15% of the total dietary fibre and was similar for all finisher diets (2.6±0.2%).

In vivo starch digestibility was high for all diets, with over 77% starch digestion in the proximal jejunum and achieving almost 100% digestion in the colon (Table 5.3). The percentage of starch digestion decreased linearly as dietary PS increased for jejunum and distal ileum sections. Proximal ileum starch digestibility showed a quadratic response with the lowest value at 100% PS. A trend ($P = 0.073$) for starch digestibility to decrease with increasing PS was found in the colon.

The pH of the crop contents responded quadratically to PS concentration, reaching an estimated minimum at the 55% PS concentration (Table 5.4). Ileum and caeca content pH did not change significantly, but both showed a trend (both $P = 0.064$) to change quadratically with PS concentration, attaining an estimated minimum at the 61% PS concentration for the ileum and a maximum for the caeca at 38% PS concentration. However, these effects were mostly driven by the higher distal ileum pH found when fed 0% PS and by the lower caecal pH measured in birds fed 100% PS.

On average, the total SCFA concentration was highest in the caeca (170 µmol/g of digesta) followed by the ileum (81.54 µmol/g) and the crop (59.19 µmol/g; Table 5.5). The amount of total, acetic, propionic and lactic acids in the crop increased in a linear fashion with increasing dietary PS, but on a proportional basis, only iso-butyric and valeric acids (linearly reduced) were affected by dietary PS. Absolute values for total and propionic acid in the distal ileum responded quadratically and linearly, respectively, to PS concentration. The total SCFA found reached an estimated maximum at 58% PS, while propionic acid increased linearly with diet PS. However, propionic acid showed a trend to respond in a quadratic fashion, reaching an estimated maximum at 68% PS ($P = 0.098$). The proportional SCFA composition was not

Table 5.3. Effect of the proportion of dietary wheat and pea starch on the cumulative percentage of apparent digested starch in broilers at 28 d of age

	Diets						Regression			SEM
	0[1]	20	40	60	80	100	*P* value	R^2	Equation	
Proximal jejunum	87.0	82.5	79.6	77.9	78.6	77.9	L=0.002	0.35	$y = -0.08x + 84.77$	1.013
Distal jejunum	95.7	92.0	89.0	88.2	89.6	85.3	L<0.001	0.59	$y = -0.09x + 94.28$	0.796
Proximal ileum	99.1	98.0	96.2	96.6	96.7	95.4	Q=0.037	0.76	$y = 3_x10^{-4}x^2 - 0.06x + 98.94$	0.264
Distal ileum	99.1	98.5	98.3	97.5	97.8	96.8	L<0.001	0.81	$y = -0.02x + 98.97$	0.160
Colon	98.3	98.2	97.9	97.5	98.3	97.6	NS	-	-	0.095

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.
P value: indicates regression *P* value; L: linear regression; Q: quadratic regression; SEM: pooled standard error of the mean; number of replications = 4 pens.

Table 5.4. Effect of the proportion of dietary wheat and pea starch on crop, ileum and caeca contents pH of broilers at 28 d of age

	Diets						Regression			SEM
	0[1]	20	40	60	80	100	*P* value	R^2	Equation	
Crop	5.19	4.89	4.92	4.84	5.00	5.01	Q=0.042	0.05	$y = 9_x10^{-5}x^2 – 0.01x + 5.14$	0.045
Ileum	6.85	6.15	6.47	6.13	6.26	6.34	NS	-	-	0.082
Caeca	6.50	6.59	6.48	6.53	6.59	6.26	NS	-	-	0.036

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.
Data was applied natural logarithm transformation; *P* value: indicates regression *P* value; Q: quadratic regression; SEM: pooled standard error of the mean; number of replications = 4 pens.

Table 5.5. Effect of the proportion of dietary wheat and pea starch on SCFA concentration (µmol/g of digesta) and composition (%) in the crop, distal ileum and caecal contents of broilers at 28 d of age

	Diets						Regression			SEM
	0[1]	20	40	60	80	100	*P* value	R^2	Equation	
Crop (µmol/g)										
Total	54.5	56.9	58.0	60.6	61.7	63.5	L=0.008	0.41	y=0.09x+54.8	0.99
Acetic acid	22.2	23.2	23.8	25.0	25.5	26.3	L<0.001	0.52	y=0.04x+22.3	0.41
Propionic acid	7.6	9.0	8.5	8.9	8.9	9.6	L=0.019	0.23	y=0.01x+8.0	0.21
Iso-butyric acid	1.4	1.4	1.4	1.4	1.3	1.3	NS	-	-	0.03
Butyric acid	-	-	-	-	-	-	-	-	-	-
Iso-valeric acid	-	-	-	-	-	-	-	-	-	-
Valeric acid	1.4	1.4	1.4	1.2	1.2	1.1	NS	-	-	0.06
Caproic acid	0.6	0.6	0.5	0.6	0.6	0.5	NS	-	-	0.02
Lactic acid	21.4	21.5	22.3	23.6	24.2	24.7	L=0.019	0.23	y=0.04x+21.07	0.56
Crop (%)										
Acetic acid	40.8	40.8	41.1	43.9	41.4	41.5	NS	-	-	0.51
Propionic acid	13.9	15.8	14.8	15.6	14.4	15.2	NS	-	-	0.37
Iso-butyric acid	2.6	2.4	2.4	2.4	2.1	2.0	L<0.001	0.41	y=-0.005x+2.6	0.06
Butyric acid	-	-	-	-	-	-	-	-	-	-
Iso-valeric acid	-	-	-	-	-	-	-	-	-	-
Valeric acid	2.5	2.4	2.5	2.1	2.0	1.7	L=0.006	0.30	y=-0.008x+2.6	0.11
Caproic acid	1.1	1.0	0.9	1.0	1.0	0.8	NS	-	-	0.04
Lactic acid	39.1	37.8	38.3	41.4	39.2	38.8	NS	-	-	0.67
Distal ileum (µmol/g)										
Total	67.2	78.8	84.2	96.7	93.4	74.7	Q=0.025	0.27	$y=-0.008x^2+1.0x+64.7$	3.82
Acetic acid	60.2	60.7	63.5	61.3	63.8	59.1	NS	-	-	1.11
Propionic acid	4.7	6.3	8.4	9.9	9.4	8.1	L=0.050	0.17	y=0.04x+5.8	0.70
Iso-butyric acid	0.5	0.6	0.9	0.4	1.2	1.0	NS	-	-	0.13
Butyric acid	-	1.7	1.5	1.0	2.2	1.4	NS	-	-	0.29
Iso-valeric acid	0.5	0.8	1.0	0.6	1.1	0.7	NS	-	-	0.12
Valeric acid	0.4	0.5	0.6	0.4	1.0	0.9	NS	-	-	0.14
Caproic acid	1.1	0.8	1.1	1.0	1.1	0.8	NS	-	-	0.10
Lactic acid	-	7.4	7.3	22.1	13.6	2.6	NS	-	-	3.45

	Diets						Regression			SEM
	0[1]	20	40	60	80	100	*P* value	R^2	Equation	
Distal ileum (%)										
Acetic acid	90.0	78.6	77.3	67.0	72.6	79.2	NS	-	-	3.10
Propionic acid	6.6	7.8	10.2	10.9	10.4	10.7	NS	-	-	0.87
Iso-butyric acid	0.7	0.6	1.0	0.4	1.3	1.4	NS	-	-	0.14
Butyric acid	-	2.0	1.6	0.8	2.3	2.0	NS	-	-	0.29
Iso-valeric acid	0.7	1.0	1.2	0.7	1.2	0.9	NS	-	-	0.14
Valeric acid	0.5	0.6	0.6	0.3	1.0	1.1	NS	-	-	0.15
Caproic acid	1.6	1.1	1.3	1.0	1.2	1.1	NS	-	-	0.12
Lactic acid	-	8.4	6.8	19.0	10.1	3.7	NS	-	-	2.87
Caeca (µmol/g)										
Total	156	159	162	166	177	203	Q=0.002	0.77	$y=0.006x^2-0.2x+159$	3.7
Acetic acid	94	94	97	98	105	126	Q<0.001	0.79	$y=0.001x^2-0.3x+96$	2.6
Propionic acid	30.7	31.6	30.8	32.9	35.2	32.7	L=0.043	0.17	y=0.03x+30.7	0.56
Iso-butyric acid	4.7	4.9	1.6	5.0	5.6	4.1	NS	-	-	0.16
Butyric acid	16.0	17.4	17.7	19.2	19.0	29.4	L=0.014	0.25	y=0.1x+14.5	1.51
Iso-valeric acid	4.6	4.7	4.5	4.9	5.3	4.2	NS	-	-	0.13
Valeric acid	1.5	4.7	4.5	4.9	5.1	4.8	NS	-	-	0.07
Caproic acid	2.0	1.9	2.0	2.0	2.0	1.5	NS	-	-	0.06
Lactic acid	-	-	-	-	-	-	-	-	-	-
Caeca (%)										
Acetic acid	60.1	59.1	60.2	58.6	59.3	62.2	Q=0.034	0.25	$y=0.0008x^2-0.07x+60.3$	0.39
Propionic acid	19.6	19.9	19.2	19.8	19.9	16.3	Q=0.039	0.34	$y=-0.0007x^2+0.05x+19.3$	0.38
Iso-butyric acid	3.0	3.1	2.9	3.0	3.1	2.1	Q=0.035	0.33	$y=-0.0002x^2+0.01x+2.9$	0.11
Butyric acid	10.3	11.0	11.0	11.6	10.7	14.2	NS	-	-	0.58
Iso-valeric acid	2.9	3.0	2.8	2.9	3.0	2.1	Q=0.025	0.39	$y=-0.0001x^2+0.007x+2.8$	0.09
Valeric acid	2.9	2.9	2.8	2.9	2.9	2.4	Q=0.008	0.48	$y=-0.0001x^2+0.007x+2.8$	0.05
Caproic acid	1.3	1.1	1.2	1.2	1.1	0.8	Q=0.027	0.44	$y=-0.00008x^2+0.005x+1.2$	0.05
Lactic acid	-	-	-	-	-	-	-	-	-	-

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.

P value: indicates regression *P* value; L: linear regression; Q: quadratic regression; NS: not significant; SEM: pooled standard error of the mean; number of replications = 12 male broilers.

affected by PS concentration, but acetic acid showed a trend ($P = 0.086$) to change quadratically with a minimum at 61% PS, while butyrate showed a trend ($P = 0.094$) to increase linearly. No lactic acid was found in the ileum of birds fed 0% PS. Although not statistically significant ($P = 0.116$), both the amount and the percentage of lactic acid in the distal ileum increased from 20 to 60% PS and then declined at higher concentrations.

The relative empty weights of the crop, proventriculus, jejunum, ileum, caeca and colon increased linearly with PS concentration, while no effect was found for the gizzard and duodenum (Table 5.6). The relative weight of digesta content for the crop, jejunum and ileum increased linearly with PS concentration as well, while no effect was found for other digestive tract sections. Increasing the concentration of PS resulted in a linear increase in the relative length of all sections of the small intestine and caeca but did not affect colon length (Table 5.6). Heart, liver and pancreas relative weights were not affected by diet PS concentration (data not shown).

The average serum concentrations of GLP-1 and PYY in 28 old broilers were 1,651.5± 1,183.9 and 361.8±65.4 pg/ml, respectively. Diet concentration of PS did not affect GLP-1 or PYY serum concentrations (Table 5.7).

The relative mRNA levels of target genes were not affected by starch type, with the exception of the expression of proglucagon in the ileum, which decreased linearly as PS concentration increased (Table 5.8).

5.5 DISCUSSION AND CONCLUSIONS

Starch digestibility values in all sections of the small intestine in the current study were high in comparison to site appropriate digestibility values for intact starch sources (Weurding et al., 2001; Karunaratne et al., 2018). The high starch digestibility was due to the semi-purified nature of the starch, and the digestibility values were similar, although slightly lower than other published research using semi-purified starch sources (Yutse et al., 1991). The purification process reduces grain-associated compounds that could reduce digestibility (Tyler, 1982). This process can also result in damage to starch granules, thereby improving enzyme accessibility. Despite high digestibility values, intrinsic characteristics of WS and PS remained, as *in vivo*

Table 5.6. Effect of the proportion of dietary wheat and pea starch on digestive tract empty weights, contents and small intestine length of broilers at 28 d of age as a percentage of body weight

	Diets						Regression			SEM
	0[1]	20	40	60	80	100	*P* value	R^2	Equation	
Empty weights (% of body weight)										
Crop	0.32	0.33	0.33	0.32	0.37	0.37	L=0.007	0.07	$y = 6_x10^{-4}x + 0.31$	0.007
Proventriculus	0.36	0.37	0.39	0.37	0.39	0.41	L=0.040	0.04	$y = 4_x10^{-4}x + 0.36$	0.007
Gizzard	1.0	1.0	1.2	1.1	1.1	1.1	NS	-	-	0.02
Duodenum	0.58	0.59	0.64	0.60	0.61	0.61	NS	-	-	0.011
Jejunum	1.28	1.34	1.35	1.32	1.42	1.42	L=0.032	0.05	y = 0.001x + 1.29	0.021
Ileum	0.93	0.95	1.02	1.02	1.03	1.04	L=0.017	0.06	$y= 6_x10^{-4}x + 0.66$	0.017
Total SI	2.8	2.9	3.0	2.9	3.1	3.1	L=0.032	0.05	y=0.003x+2.8	0.59
Caeca	0.32	0.32	0.36	0.34	0.37	0.36	L=0.022	0.06	$y = 4_x10^{-4}x + 0.32$	0.006
Colon	0.086	0.087	0.089	0.090	0.095	0.102	L=0.002	0.09	$y = 2_x10^{-4}x + 0.084$	0.0017
Length (cm/100 g of BW)										
Duodenum	1.59	1.64	1.61	1.69	1.80	1.66	L=0.038	0.05	y = 0.001x + 1.60	0.021
Jejunum	3.66	3.94	3.86	3.97	4.25	4.19	L<0.001	0.14	y = 0.005x + 3.72	0.048
Ileum	3.64	3.97	4.03	4.06	4.26	4.29	L<0.001	0.14	y = 0.006x + 3.74	0.055
Total SI	8.9	9.6	9.5	9.7	10.3	10.1	L<0.001	0.15	y = 0.01x + 9.1	0.49
Caeca	1.52	1.59	1.61	1.58	1.73	1.70	L=0.004	0.08	y = 0.002x + 1.53	0.022
Colon	0.26	0.28	0.27	0.26	0.28	0.28	NS	-	-	0.005
Contents (% of body weight)										
Crop	0.50	0.72	0.66	0.91	1.17	1.45	L<0.001	0.17	y = 0.009x + 0.44	0.079
Proventriculus	0.05	0.06	0.07	0.05	0.10	0.10	NS	-	-	0.010
Gizzard	0.7	0.5	0.8	0.7	0.6	0.7	NS	-	-	0.04
Duodenum	0.23	0.22	0.20	0.21	0.24	0.21	NS	-	-	0.006
Jejunum	0.81	0.91	0.91	1.09	1.31	1.23	L<0.001	0.31	y = 0.005x + 0.79	0.032
Ileum	0.76	0.81	0.87	1.03	0.98	0.99	L<0.001	0.13	y = 0.003x + 0.78	0.026
Total SI	1.8	1.9	2.0	2.3	2.5	2.4	L<0.001	0.25	y = 0.008x + 1.8	0.62
Caeca	0.27	0.23	0.26	0.22	0.32	0.28	NS	-	-	0.011
Colon	0.05	0.07	0.06	0.05	0.06	0.06	NS	-	-	0.003

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.

P value: indicates regression *P* value; L: linear regression; NS: not significant; SEM: pooled standard error of the mean; number of replications = 4 pens.

Table 5.7. Effect of the proportion of dietary wheat and pea starch on GLP-1 and PYY serum concentrations (pg/mL) of broilers at 28 d of age

	Diets						Regression			SEM
	0[1]	20	40	60	80	100	P value	R^2	Equation	
GLP-1	1650	1478	1964	1995	1277	1545	NS	-	-	141.5
PYY	369	366	332	368	375	361	NS	-	-	7.8

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet. *P* value: indicates regression *P* value; NS: not significant; SEM: pooled standard error of the mean; number of replications = 12 male broilers.

Table 5.8. Effect of the proportion of dietary wheat and pea starch on relative[1] mRNA levels of proglucagon, proglucagon-B and peptide tyrosine-tyrosine in jejunum and ileum samples of broilers at 28 d of age

	Diets						Regression			SEM
	0[2]	20	40	60	80	100	*P* value	R^2	Equation	
Jejunum										
PG	0.9	1.3	0.5	1.0	0.8	0.6	NS	-	-	0.09
PGB	0.9	1.3	0.4	0.9	0.6	0.3	NS	-	-	0.13
PYY	1.2	1.5	0.8	1.0	1.0	1.0	NS	-	-	0.08
Ileum										
PG	1.5	1.5	1.3	1.3	1.1	1.3	L=0.047	0.08	y= -0.001x + 1.2	0.05
PGB	0.9	0.9	0.7	0.9	0.7	0.8	NS	-	-	0.04
PYY	0.9	0.7	0.8	0.7	0.7	0.7	NS	-	-	0.07

[1] Obtained values of proglucagon, proglucagon B and PYY were normalized by the average of GAPDH and RPS7

[2] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet. PG: proglucagon; PGB: proglucagon-B; PYY: peptide tyrosine-tyrosine; *P* value: indicates regression *P* value; L: linear regression; NS: not significant; SEM: pooled standard error of the mean; number of replications = 12 male broilers.

digestibility decreased as PS replaced WS in the diets. Statistical differences in starch digestibility were found for all areas of the small intestine, but the degree of difference decreased in more distal sections (From proximal jejunum to distal ileum the difference in the percentage of starch digestibility was: 9.1, 10.4, 3.7 and 2.3%). The separation between starch digestibility values was further reduced in the colon (0.7%) where statistical differences disappeared, suggesting that any starch remaining in the distal ileum had been fermented (Bird et al., 2007). Therefore, starch digestibility values confirmed the potential of the dietary treatments used in this study to have activated L-cells.

The digesta content pH in this trial was measured as an initial indication of fermentation, under the assumption that lower pH values indicate higher SCFA concentration. The results indicate that only crop pH was significantly affected by treatment in a quadratic fashion. However, the other sections measured, distal ileum and caeca, showed strong quadratic trends (both $P = 0.064$). Production of SCFA, as well as CO_2 and H^+, are mainly associated with fermentable carbohydrate intake (e.g. Józefiak et al., 2004; den Besten et al., 2013; Olnood et al., 2015). However, during the fermentation process, other nutrients are fermented as well, such as proteins, and the pH is the result of the mixture of resulting products. Proteolytic fermentation, for instance, produces not only SCFA but also NH_3, amines, volatile phenols and indoles, which are toxic and also affect pH (Williams et al., 2001). Other factors such as passage rate can also affect pH values (Potkins et al., 1991; Gordon and Roland, 1997). It is possible that differences in the composition of the bacterial community as a result of a differing composition of the digesta, a reduced passage rate and a different rate of protein fermentation in this trial, could have resulted in a lack of correlation between some of the SCFA results and pH. In fact, a similar trial run in laying hens resulted in a linear decrease in both crop and distal ileum pH as PS concentration increased (Chapter 6).

Concentration of PS directly affected total SCFA concentration in all three sections measured: crop, ileum and caeca. In the crop, it was found that the concentration of SCFA increased with PS level, but the proportion of the major acids (acetic, propionic and lactic) was maintained, while minor acids (isobutyric and valeric) decreased. Branched SCFA are associated with protein fermentation (Macfarlane et al., 1992), and consequently, these results suggest that as PS concentration increases, protein fermentation decreases. As the total amount of starch in diets was identical, the increase in total SCFA concentration could be an indication of longer retention time in the crop, which is supportive of activation of the ileal brake. The gizzard regulates the flow of digesta in the upper digestive tract (Chaplin et al., 1992). As the ileal brake is activated, particularly the release of PYY, results in a reduction of gastric emptying and a decrease of passage rate (Savage et al., 1987; Chen et al., 1997). Thus, an indication of longer retention time in the gizzard is supportive to ileal brake activation.

In the distal ileum, a quadratic response was found for total SCFA, which is supported by the quadratic trend found for distal ileum pH. However propionic acid, which was the only acid

that changed significantly with treatment, increased linearly with PS, with a trend to change quadratically. In fact, numerical differences appear to be driven by the amount of lactic acid found in the distal ileum, but due to a high level of variability, this was not statistically significant. The quadratic response found on ileal SCFA was unexpected as *in vivo* starch digestion showed a linear increase in the remaining starch content in distal ileum digesta as PS concentration increased. Based on digestibility levels and the amount of starch in each finisher diet, remaining starch in the distal ileum increases from 0.43 to 1.43 g of starch/100g of digesta for 0 to 100% PS. In fact, data from laying hens showed a linear decrease in distal ileum pH supporting the digestibility data (Chapter 6). However, it is important to remember that laying hens have a more mature digestive tract and the enteric microbial community will have evolved to the treatment diets for 20 weeks before digesta collection, while the digestive tract and microbial community of broilers might not be completely developed when tissue collection occurred at 28 d of age (Gong et al., 2007). Nevertheless, the quadratic response in distal ileum SCFA is supported by unpublished broiler data from our lab (Savary, unpublished). Under a similar experimental conditions, distal ileum digesta collected from 25 d old broilers fed graded level of dietary PS (0, 25, 50, 75 or 100% PS) and challenged or not with 30x label dose of Coccivac-B52 showed a significant quadratic effect on the total SCFA found in this section (P = 0.006) regardless of challenge. It is possible that as PS increases, a shift in enteric microbiota towards lactic acid consuming species could result in a decrease of the amounts found for both lactic acid and total SCFA in the distal ileum. Alternatively, under similar fermentation levels, the higher digesta content found in the distal ileum could result in a decrease in concentration of SCFA for that section due to a dilution effect. Likewise, as both distal ileum and caeca empty weights increased with PS concentration, it is possible that the absorption rate of SCFA is increased, again resulting in a quadratic effect as SCFA concentration decreases.

The amount of SCFA found in the distal ileum in this study are higher than those reported by Józefiak et al. (2006). In their study, where the effects of beta-glucanase were assessed in barley or oat-based diets fed broilers, they found similar concentrations of lactic acid in the distal ileum, but no propionic or butyric acid, while acetate values were approximately only 10% of the amounts reported here. In another study that investigated the effect of modified corn or soybean meal on broilers, no lactic acid was reported. However, the amounts of acetate, propionate and butyrate were very similar to those reported here (38.62, 2.38 and 1.19 µmol/g respectively)

although acetate was a little bit lower (Czerwiński et al., 2017). The mixture composition of distal ileum SCFA was not affected by PS concentration in the current study, but acetic acid showed a trend to decrease quadratically up to 61% PS concentration, while butyrate showed a trend to increase linearly. Again, although not significant due to variability, numerical changed in lactic acid percentage suggest a quadratic effect of PS, with a maximum value around the 60% PS concentration. These changes suggest a shift in carbohydrate-fermenting bacteria in the distal ileum as concentration of PS increases.

Total caecal SCFA showed a quadratic response with PS. However, this effect was driven by a sudden increase in the amount of SCFA found in birds fed 80 and 100% where total SCFA amount increased 13 and 30%, respectively, when compared to 0% PS. Starch digestibility showed that most of the starch had been digested or fermented before entering the caeca, except for the highest concentrations of PS. Changes in the SCFA composition in this section of the digestive tract suggest an effect of PS concentration on the type of bacteria present in the caeca. In fact, concentration of acids associated to protein fermentation (iso-butyric, iso-valeric and valeric acid; Williams et al., 2001) decreased in 100% PS, while others associated with starch fermentation (acetic and butyric acid; Williams et al., 2001) increased. The amount of SCFA found in this study are very similar to those previously reported (Józefiak et al., 2006; Czerwiński et al., 2017; Wang et al., 2017). Previous literature is consistent in the lack or minimal amount of lactic acid found in chicken caeca and the predominance of acetate. Like SCFA, pH responded quadratically to PS concentration, but again this effect was likely driven by the lower pH value found in 100% PS fed birds, which had the highest amount of SCFA. No effect of dietary PS was found in laying hen caecal digesta pH (Chapter 6). Although differences in the laying hen and broiler data could be differences due to maturity or selection pressures, the lack of change in laying hen caecal pH is consistent with broiler starch digestibility values, and it could suggest that all starch was completely digested and/or fermented by the end of the distal ileum.

Evidence in mammals shows that SCFA have the capacity of activating L-cells (Cani et al., 2007; Kaji et al., 2011; Psichas et al., 2015b). Research in chickens seems to be consistent with the findings in mammals, indicating that SCFA can activate L-cells also in this species (De Maesschalck et al., 2015; Onrust et al., 2015). As L-cells are located in the jejunum and ileum,

starch digestibility, pH and SCFA results indicate that there is potential for L-cell activation in the broiler distal small intestine with the inclusion of dietary PS.

The proportional weight of every section of the digestive tract with the exception of gizzard and duodenum increased linearly with PS concentration. Crop contents remarkably increased with PS, which probably promoted crop muscle development in order to hold and evacuate digestive contents into the esophagus and proventriculus (Chaplin et al., 1992), explaining the increase in crop weight with PS. As the digesta content in jejunum and ileum also showed a linear increase, the increase in empty weights of these sections could also be related to muscle development. However, these tissues were also longer. Similar results have been found for the pig's colon when animals are fed resistant starch (Topping et al., 1997; Bird et al., 2007). Bird et al. (2007) found a direct relationship between pig colon length and the amount of amylose present in the diet. These results are supportive of L-cell involvement. Along with GLP-1, L-cells contain another peptide that belongs to the glucagon superfamily known as glucagon-like peptide-2 (GLP-2) and that it is co-secreted with GLP-1 (Burrin et al., 2003). Intestinal growth is promoted by GLP-2 as a consequence of nutrient sensing (Tsai et al., 1997; Ghatei et al., 2001; Burrin et al., 2003), making it a potential player in the increase of jejunum and ileum weight and length with increased dietary PS. An increase in empty weight and length of the caeca was also observed with increasing concentration of PS. Although no PYY or GLP-1 immunoreactive cells have been reported in this section, there is the possibility that these peptides could be affecting caecal length and weight through a paracrine mechanism. Alternatively, butyrate has been associated with intestinal health by promoting the growth of the digestive tract wall and decreasing inflammation (Onrust et al., 2015). As the amount of butyrate found in the caeca increased with PS concentration, it is possible that the observed changes in weight and length or the caeca are associated with this acid.

Although no differences in feed intake or feeding behaviour were observed (Chapters 3 and 7), crop content increased with dietary PS, supporting a reduction in gastric emptying, which might suggest ileal brake activation. The gizzard is the organ that regulates the flow of feed from the crop into the rest of the digestive tract (Chaplin et al., 1992). Neuropeptides GLP-1 and PYY both have been shown to reduce gastric emptying in mammals when nutrients are sensed by L-cells (Savage et al., 1987; Schirra and Göke, 2005); the linear increase in crop content could be

an indication of a lower rate of gastric emptying as a result of L-cell activation. Jejunum and ileum relative digestive content also increased with PS concentration. DF increased 3.4% from 0% PS to 100% PS in finisher diets. The relative increase in fibre material, along with the remaining amount of undigested starch and potential differences in water holding capacity could explain the change in small intestine content with PS concentration, without affecting feed intake (Hetland and Svihus, 2001). Thus, although the presence of a higher level of dietary PS could be partially responsible for the increase in small intestine contents, the increase in total DF incorporates a confounding factor that makes interpretation difficult.

No effect of PS on GLP-1 or PYY venous concentration levels was found. It is possible that this could be the result of collecting blood from the brachial vein instead of the portal vein or arterial blood. These peptides are released from the digestive tract enteroendocrine cells into the bloodstream. Their effects seem to be the result of both endocrine and paracrine action through the activation of the vagus nerve (Abbott et al., 2005). Thus, collection site could be responsible for the lack of effect. However, gene expression analysis did not show an effect of PS on either proglucagon or PYY RNA expression levels, with the exception of a linear decrease in ileum proglucagon gene expression. Although it is important to remember that proglucagon is a gene yields more than GLP-1, the lack of response in proglucagon B and the decrease in proglucagon in the jejunum with an increase in dietary PS do not seem to support activation of ileal brake by PS. This lack of effect seems to be contrary to all the effects previously mentioned that support L-cell activation. A key difference between chickens and mammals is the feeding schedule. While mammals eat discrete meals, broilers are fed ad-libitum. As a consequence, with the possible exception of the time at the end of an extended dark period (>8 h), broilers always have feed in the digestive tract (Veerkamp, 1986; Sengor et al., 2006; Shynkaruk, 2017). As a result of this, nutrient sensing mechanisms could be continuously activated. A review of the literature has failed to provide the normal serum concentrations of GLP-1 in chickens. The average concentration found in this study (1652 pg/mL or 496 pmol/L) seems high when compared to normal concentration levels reported in mammals (8-40 pmol/L; Massimino et al., 1998; Wachters-Hagedoorn et al., 2006; Vollmer et al., 2008). Thus, it is possible that, similar to insulin, broilers present some type of GLP-1 resistance, and so no effects on passage rate or feed intake are observed. Contrary to these findings, serum GLP-1 and PYY concentrations in laying hens (average serum concentration of 698 and 427 pg/mL each) were found to be affected by PS

concentration by increasing linearly and changing in a quadratic fashion, respectively (Chapter 6). Maturity or divergent performance selection pressures could be the reason for the different GLP-1 and PYY response in broilers and laying hens to PS concentrations.

In conclusion, this study found evidence of the presence of L-cell activators, starch and SCFA, in the distal small intestine that were affected by PS concentration level. Also, dietary PS promoted digestive tract tissue development and provided indirect evidence of reduced gastric emptying. However, no direct evidence of ileal brake activation was found, either by ELISA or gene expression analyses and hence other potential mechanisms should be explored to explain the observed improvement in performance when broilers are fed sources of slowly digested starch.

6.0 Assessing the effect of rate and extent of starch digestion on ileal brake activation in laying hens

Research presented in Chapter 5 examined both potential and direct evidence of L cell activation. However, direct evidence of ileal brake activation was not conclusive in broilers (serum and gene expression levels of GLP-1 and PYY). Although broilers and laying hens are the same species, selective pressures, as well as other differences, such as maturity and length of exposure to the diets, may produce different physiological responses to diets varying in starch digestion characteristics. The objective of Chapter 6 was to evaluate the effects dietary pea starch inclusion level on the digestive tract characteristics and activation of the ileal brake in laying hens by measuring digestive tract parameters (empty weight, length, content and pH), feed passage rate, and GLP-1 and PYY status (gene expression, serum concentrations). This portion of the research was done concurrently with performance data presented in Chapter 4.

6.1 ABSTRACT

The inclusion of starch with lower digestion rate and extent has proven to be beneficial in laying hen production. However, data is lacking on how starch digestion affects digestive tract characteristics and activation of the ileal brake. To answer these questions, six diets were formulated to contain differing ratios (0:100, 20:80, 40:60, 60:40, 80:20 and 100:0) of semi-purified wheat (WS; rapidly digested) and pea (PS; slowly digested) starch and each diet was fed for 20 weeks to 72 Lohmann LSL-lite hens housed in conventional cages in replication groups of 12 birds. At the end of the experiment, feed passage rate was measured using TiO_2 as a marker and by collecting excreta every 30 min for 13 h. Subsequently, blood samples and tissues were collected for measuring serum GLP-1 and PYY, *in situ* digesta pH, and weights of digestive tract sections and other organs (heart, liver, pancreas). Data were analyzed with regression analysis, and differences were accepted as significant when $P \leq 0.05$. A quadratic effect of PS concentration on empty gizzard and crop contents was found with an estimated maximum at 53 and 50% PS, respectively. Gizzard to ileum digesta content, as well as ileum and caecal length, increased linearly with PS concentration. Crop and ileum pH decreased linearly with higher PS concentration suggesting increased starch fermentation. Pancreas weight increased linearly with PS level. Concentration of dietary PS did not affect feed passage rate. Serum GLP-1 concentration increased linearly with PS while PYY changed quadratically with an estimated maximum at 34% PS. Changes in digestive tract morphology and content, and serum GLP-1 and PYY concentrations are suggestive of L-cell activation and the resulting action of PYY and GLP-2. In conclusion, activation of the ileal brake elements may play a role in producing the effect of PS concentration observed on laying hen performance.

6.2 INTRODUCTION

The inclusion of starch with a lower rate and extent of digestion in poultry diets has been shown to have beneficial effects on poultry performance. In 2003, Weurding et al. showed that broiler feed efficiency responded favourably to the utilization of pea-corn, a more slowly digested starch source, instead of tapioca corn, which is more rapidly digested. These findings were later corroborated by others (e.g., Enting et al., 2005; Gutierrez del Alamo et al., 2009;

Chapter 3) using a variety of starch sources. Little research in this area has been conducted in laying hens, but a recent study (Chapter 4) compared the effects of feeding diets where pea starch (PS; slowly digested) replaced wheat starch (WS; rapidly digested) on a graded basis on laying hen performance. Egg production increased linearly with PS concentration, while feed:egg mass ratio was lowest at the 26% level of PS inclusion.

The mechanism(s) whereby slowly digested starch affects performance is not established, but may include changes in post-prandial metabolism due to differences in glycemic index, beneficial effects of starch fermentation, a sparing effect on amino acid oxidation and ileal brake activation. Highly digestible starch is associated with a high glycemic index, which results in a substantial and rapid increase in insulin, at least in meal-fed animals, along with storage of excess glucose as glycogen and fat (Regmi et al., 2011a; Deep, 2018). When starch is more slowly digested, the release and absorption dynamics change in that it provides a slow and continuous release and absorption of glucose, while maintaining insulin at intermediate concentrations for a more extended period of time (Seal et al., 2003). A lower but continuous glucose concentration rise allows more time for protein deposition and more direct use of nutrients, along with a less demanding regulation of insulin-glucose blood concentrations (Weurding et al., 2001). Regarding fermentation, any non-absorbed starch can be subjected to enteric bacteria metabolism, leading to the production of short-chain fatty acids (SCFA), among other products, which ultimately can decrease the pH of luminal content (Apajalahti et al., 2004). The SCFA can be sensed by enteroendocrine cells as well as being used as an energy source by enterocytes (Macfarlane and Macfarlane, 2003). The presence of glucose or higher concentrations of SCFA in the distal small intestine would increase the availability of energy sources for enterocytes of that section of the digestive tract, which could partially spare glutamine or glutamate oxidation for that purpose (Watford et al., 1979). At the same time, both glucose and SCFA present in the distal small intestine could be sensed by the enteroendocrine L-cells (Honda et al., 2017), activating a mechanism known in mammals as the ileal brake. Little is known about this mechanism in chickens, but research in mammals has shown that activation of L-cells reduces digesta passage rate, potentially improves digestion efficiency, increases satiety and thereby reduces feed intake.

When broilers were fed graded levels of pea starch (PS) for 28 d, physiological changes were observed which were potentially linked to improved feed efficiency (Chapters 3 and 5). These changes included a linear increase in the weight and length of the digestive tract, as well as digesta weights in the crop, jejunum, and ileum with increasing PS. Analysis of SCFA showed that carbohydrate fermentation increased linearly in the crop. Ileum SCFA concentration reached a maximum at 57% PS, while minimal and maximum values in caeca were reached at 18 and 100% PS, respectively. These effects suggest a potential reduction of passage rate in the proximal digestive tract allowing for more fermentation in the crop as PS concentration increases. They also suggest that much of the fermentable starch disappears by the time the digesta reaches the caeca. Fermentation, as well as the growth of intestinal tissue, could imply L-cell involvement. However, gene expression and serum concentrations of GLP-1 and PYY, the two neuropeptides believed to be responsible for the activation of this mechanism failed to confirm this effect. Only proglucagon, the precursor of GLP-1, gene expression in the ileum responded to dietary PS; however, it decreased as dietary PS concentration increased. Thus, although no mechanism has been defined so far, it is of interest to determine if the positive effects of feeding PS observed in laying hens production are accompanied by similar physiological and morphological changes.

The objectives of this study were to evaluate the effect of rate and extent of starch digestion on physiological and morphological changes in the laying hen digestive tract, with emphasis on activation of the ileal brake. It was hypothesized that increasing dietary PS would result in more carbohydrate fermentation in the distal small intestine, which will be reflected by lower pH values. The presence of glucose and SCFA in the distal small intestine will also activate L-cells, increasing serum GLP-1 and PYY as well as decreasing passage rate.

6.3 MATERIALS AND METHODS

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (Canadian Council on Animal Care, 1993, 2009).

6.3.1 *Experimental treatments*

To study the effect of starch digestion rate and extent in laying hens, six treatment diets were formulated to be nutritionally identical (Table 6.1) with the exception of the proportion of starch type. Semi-purified WS (Archer Daniels Midland Company, Montreal, QC, Canada) and PS (Parrheim Foods, Saskatoon, SK, Canada) were used after *in vitro* analysis confirmed that WS was digested more rapidly and to a larger extent than PS (Chapter 3). The purity of the starch sources was not equal (WS = 92.5% *vs*. PS = 80.0%), and therefore purified pea protein (Parrheim Foods, Saskatoon, SK, Canada) was added to the WS to equalize starch, protein and fibre levels. The starch fraction of the 0% PS diet contained a mixture of 87% WS and 13% pea protein. For other diets, the appropriate amount (20, 40, 60, 80 or 100%) of the WS-pea protein mixture was replaced by PS. All diets were pelleted, and processing temperature did not surpass 85°C. Diets were manufactured at the Canadian Feed Research Centre (North Battleford, SK, Canada). Pellet Durability Index of the diets was tested using the Holmen Pellet Durability Tester (Borregaard, UK Ltd. LT 218; Payne et al., 2001). It resulted in the following (from 0 to 100% PS) values: 92, 91, 94, 87, 81 and 90% (P>0.05). The lower rate and extent of PS digestibility in comparison to WS was confirmed through *in vivo* assessment in broilers at 28 d of age (Chapter 5).

6.3.2 *Dietary analyses*

Diets were analyzed for starch (TS), moisture, fat, crude protein (CP), ash and dietary fibre (DF), primarily based on AOAC (2006) methods. Starch was determined using the Megazyme Total Starch Kit (Megazyme Inc., Chicago, IL, USA) following the method 996.11. Moisture was measured following method 930.15. Fat content was determined by ethyl ether extraction using Goldfish Extraction Apparatus (Labconco model 35001; Labconco, Kansas, MO, USA) following AOAC method 920.39. Crude protein of the diets was determined using a Leco N analyzer (Leco FP-528, Leco Corp., St Joseph, MI, USA) following method 990.03 and 6.25 was used to convert N to CP. Ash was measured (method 942.05) using a muffle oven (Lindberg/Blue BF51842, Asheville, NC 28804, USA). The Megazyme Total Dietary Fibre kit (Megazyme Inc., Chicago, IL) was used to measure soluble and insoluble DF (method 985.29).

Table 6.1. Ingredient composition of treatment diets

Ingredients (%)	0[1]	20	40	60	80	100
Wheat starch	46.57	37.25	27.94	18.63	6.52	0.00
Pea protein	6.85	5.48	4.11	2.74	0.96	0.00
Peas starch	0.00	10.68	21.37	32.05	45.94	53.42
Soybean meal	26.30	26.30	26.30	26.30	26.30	26.30
Porcine meal	5.00	5.00	5.00	5.00	5.00	5.00
Oat hulls	2.00	2.00	2.00	2.00	2.00	2.00
Soybean oil	1.51	1.51	1.51	1.51	1.51	1.51
Limestone	9.65	9.65	9.65	9.65	9.65	9.65
Monocalcium phosphate	0.70	0.70	0.70	0.70	0.70	0.70
Sodium chloride	0.34	0.34	0.34	0.34	0.34	0.34
Vitamin/mineral premix[2]	0.50	0.50	0.50	0.50	0.50	0.50
Choline Chl.	0.10	0.10	0.10	0.10	0.10	0.10
DL-Methionine	0.43	0.43	0.43	0.43	0.43	0.43
L-Threonine	0.05	0.05	0.05	0.05	0.05	0.05
Calculated composition (%)						
AME (kcal/kg)	2,800	2,800	2,800	2,800	2,800	2,800
Dry matter	89.79	89.79	89.79	89.79	89.79	89.79
Crude protein	19.35	19.35	19.35	19.35	19.35	19.35
Didestible Lys	1.17	1.17	1.17	1.17	1.17	1.17
Digestible Met	0.58	0.58	0.58	0.58	0.58	0.58
Digestible Met+Cys	0.84	0.84	0.84	0.84	0.84	0.84
Digestible Thr	0.74	0.74	0.74	0.74	0.74	0.74
Calcium	4.10	4.10	4.10	4.10	4.10	4.10
Available phosphorus	0.42	0.42	0.42	0.42	0.42	0.42

[1]Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.

[2]Supplied per kilogram of diet: vitamin A (retinyl acetate + retinyl palmitate), 8000 IU; vitamin D_3, 3000 IU; vitamin E (dl-α-topheryl acetate), 25 IU; menadione, 1.5 mg; thiamine, 1.5 mg; riboflavin, 5.0 mg; niacin, 30 mg; pyridoxine, 1.5 mg; vitamin B_{12}, 0.012 mg; pantothenic acid, 8.0 mg; folic acid, 0.5 mg; biotin, 0.06 mg; copper, 5 mg; iron, 80 mg; manganese, 80 mg; iodine, 0.8 mg; zinc, 80 mg; selenium, 0.3 mg; calcium carbonate, 500 mg; ethoxyquin, 0.625 mg; wheat middlings, 3822.79 mg.

6.3.3 *Birds and bird housing*

At 16 weeks of age, Lohmann LSL pullets were randomly allocated in groups of six and housed in 72 Specht conventional layer cages (503 cm^2/bird, 60 cm feeder trough, one lubing nipple drinker). Cages were located in the same tier, and two adjacent cages were considered an experimental unit, resulting in six replications of 12 hens per dietary treatment, arranged in a

completely randomized design. The barn was maintained at 21°C, and birds were provided with 14 h of light (10 lux) per d (with dusk and dawn periods of 15 min each); feed and water were provided *ad libitum*. Birds were fed an age-ppropriate commercial diet until the trial started at 26 wk of age. The trial lasted 20 wk and it was run concurrently with the performance trial (Chapter 4).

6.3.4 *Rate of food passage determination*

At 46 wk of age, feed passage rate was determined following a modified version of the procedure described by Salih et al. (1991). Briefly, after removing feed overnight (10 h) to avoid any night feeding that could affect the results, birds were fed a known amount of test diets with 0.3% TiO_2 addition on top for 2 h, returning them to TiO_2-free diets afterward. The remaining TiO_2 feed was weighed to determine feed consumption with the marker on a replication basis. Excreta was collected every 30 min by placing clean aluminum trays under each cage and collecting the full amount of excreta in individual bags at each collection time. Excreta collection continued for 13 h and samples were individually weighed and frozen for future titanium determination.

Excreta **s**amples were dried at 55°C using a forced air oven, weighed and ground. Titanium concentration in excreta and diet samples was determined following the procedure described by Myers et al. (2004). Briefly, 0.5 g of sample were digested along a CT-37 Kjeldahl tablet (3.5 g of K_2SO_4 + 0.4 g of $CuSO_4$; Fisher Scientific, Geel, Belgium) and 13 mL of H_2SO_4 at 420°C for 2 h. After cooling of samples for 30 min, 10 mL of 30% H_2O_2 were added and further cooling was allowed. Next, and after adding distilled water, any precipitate was removed by using filtration paper (Whatman 541, GE Healthcare, Buckinghamshire, UK). Titanium concentration was determined by measuring absorbance at 410 nm and comparing this value to a standard curve previously built using known amounts of titanium concentration. Results were expressed as a percentage of intake over 2 h. As total titanium excretion in three experimental units did not surpass 50% of the consumed TiO_2, rate of passage was calculated as the time required for 30% of titanium appearance in excreta. Recovery values were calculated by finding the best fitting curve for the data for each cage. Quadratic function resulted in the best fitting ($R^2 \geq 0.98$). The obtained equation was used to calculate the number of h required for 30% of marker to be excreted.

6.3.5 *Digestive tract assessment*

At 46 weeks of age, and 2 d after excreta collection, four birds per experimental unit were individually weighed and euthanized via T61 Euthanasia solution (0.35ml/kg body weight. Merck & Co. Inc., Kirkland, QC) intravenous injection. The digestive tract was removed and sectioned from crop to colon; jejunum and ileum were partitioned into proximal and distal sections. After removing fat, full and empty weights of each section and lengths of the small intestine, caeca and colon were recorded, along with heart, liver and pancreas weights. *In situ* pH was assessed in crop, gizzard, ileum and caeca contents prior to digestive tract removal using a pH Meter (model Phi 34, Beckman Instruments, Inc.; Fullerton, CA, USA).

6.3.6 *Determination of GLP-1 and PYY serum concentrations*

Blood was collected from the brachial vein into 1.5 mL EDTA blood collection tubes from two additional birds per experimental unit for measuring GLP-1 and PYY serum concentrations. Samples were allowed to clot and centrifuged for 15 min at 1000 x g. Resulting serum was stored (-20ºC) until further analysis. Serum concentrations of GLP-1were measured using a Chicken GLP-1 ELISA kit (Catalog No: E-EL-Ch0160; Elabscience Biotechnology, Wuhan, China). Serum concentrations of PYY were determined using a Chicken Peptide YY ELISA kit (Catalog No: CSB-EL019128CH, Cusabio Biotechnology, Wuhan, China).

6.3.7 *Statistical analyses*

Normality of the residuals and homogeneity of variance was checked in all collected data previous to statistical analysis. Data was transformed when required to meet the assumptions. Data were analyzed by regression analyses (Proc reg to test for linear relationships, and Proc Rsreg to test for quadratic relationships) using SAS 9.4 (SAS Institute, 2004). The digestive tract, passage rate and ELISA data were log+1-transformed before analysis to comply with statistical assumptions. In addition, a contrast was added to the passage data to test whether the presence of PS affected passage rate by comparing 0 *vs*. the average of 20, 40, 60, 80 and 100% PS. All differences were considered at $P \leq 0.05$ and trends were considered when $0.10 \geq P > 0.05$.

6.4 RESULTS

The effects of dietary treatment on digestive tract data on an as-is basis are shown in Table 6.2. Gizzard and jejunum empty weights changed in a quadratic fashion with PS concentration, reaching an estimated maximum and minimum at 53 and 41% PS inclusion, respectively, while ileum weight increased linearly with PS. Total small intestine empty weight increased linearly with PS, but it also demonstrated a quadratic trend ($P = 0.058$) with an estimated minimum at 34% PS. Ileum and caeca length increased linearly with concentration of PS. Total small intestine length increased by 7 cm from 0 to 100% PS. Diet inclusion of PS affected crop content in a quadratic manner with an estimated maximum at the 50% PS concentration. Gizzard, duodenum, jejunum, ileum and total small intestine content increased linearly with PS, resulting in a 33, 40, 50, 57 and 45% increase, respectively, between the 0 to 100% PS treatments.

An inverse linear relationship was found between PS concentration and the pH of crop and ileum contents, decreasing from 5.06 to 4.79 and 6.65 to 6.29, respectively (Table 6.3). No effect of dietary starch was found on gizzard or caeca pH. Pancreas weight linearly increased 18% between 0 to 100% PS treatments. No other effects of diet on organ weights were noted (Table 6.3).

No dietary effect was found for feed passage rate, with an average 5.28 h required for 30% of consumed marker to be excreted (Table 6.4). However, a planned contrast between 0% PS and the average of all other treatments showed a trend for the inclusion of PS in the diet to decrease passage rate (4.61 vs. 5.41 h, $P = 0.051$).

The presence and level of PS affected serum concentrations of both GLP-1 and PYY at the end of the experiment (Table 6.5). GLP-1 increased linearly with PS inclusion level while PYY showed a quadratic effect with an estimated maximum at 34% PS concentration.

6.5 DISCUSSION AND CONCLUSIONS

Diets were formulated to provide an equal amount of starch for each treatment. However, a lower starch content in the semi-purified PS used to make the diets, when compared to the samples tested for formulation purposes, resulted in a gradual reduction of the diet starch concentration as PS inclusion increased. The reduction in starch also resulted in a proportional

Table 6.2. Effect of the proportion of dietary wheat and pea starch on digestive tract empty weight, digesta content, and small intestine length of laying hens at 46 weeks of age

	Diets						Regression		SEM
	0[1]	**20**	**40**	**60**	**80**	**100**	***P* value**	**R^2**	**Equation**	
Body weight (g)	1678	1681	1733	1854	1756	1729	NS	-	-	17.6
Empty weight (g as-is basis)										
Crop	6.7	7.0	6.9	7.9	7.7	7.5	NS	-	-	0.19
Proventriculus	5.8	5.8	6.1	6.2	6.0	6.2	NS	-	-	0.08
Gizzard	10.2	9.8	12.0	12.7	11.0	10.0	Q<0.001	0.15	$y = -8_x10^{-4}x^2 + 0.09x + 9.6$	0.21
Duodenum	8.6	8.7	8.3	8.4	8.8	8.9	NS	-	-	0.11
Jejunum	15.9	15.4	14.7	14.7	16.3	16.9	Q=0.028	0.07	$y = 3_x10^{-4}x^2 – 0.05x +15.9$	0.28
Ileum	13.2	13.9	13.1	14.0	14.6	15.2	L=0.012	0.06	$y = 0.02x + 13.0$	0.26
Total small intestine	37.6	38.0	36.2	37.1	40.0	41.0	L=0.038	0.04	$y = 0.03x + 36.6$	0.06
Caeca	7.2	7.2	7.0	7.6	7.3	7.4	NS	-	-	0.15
Colon	2.0	2.0	1.9	1.9	1.9	1.8	NS	-	-	0.04
Length (cm)										
Duodenum	23.6	24.0	24.3	24.7	24.8	24.3	NS	-	-	0.19
Jejunum	53.1	53.0	52.6	52.6	53.8	56.2	NS	-	-	0.50
Ileum	49.7	49.8	48.6	50.9	51.8	52.7	L=0.031	0.04	$y = 0.03x + 48.8$	0.54
Total small intestine	126.4	126.8	125.4	128.2	130.3	133.2	L=0.026	0.05	$y = 0.07x + 125.0$	1.05
Caeca	23.8	23.7	24.9	26.9	26.0	24.8	L=0.036	0.04	$y = 0.02x + 24.0$	0.32
Colon	4.2	3.9	3.9	4.2	4.1	3.9	NS	-	-	0.08
Content (g as-is basis)										
Crop	13	18	19	25	14	11	Q=0.010	0.06	$y = -0.005x^2 + 0.45x + 10$	1.8
Proventriculus	0.9	0.6	0.8	0.8	0.8	0.7	NS	-	-	0.05
Gizzard	3.9	4.9	4.6	5.5	5.6	5.2	L=0.029	0.04	$y = 0.01x + 4.3$	0.21
Duodenum	2.5	3.0	3.0	3.0	3.7	3.5	L<0.001	0.10	$y = 0.01x + 2.6$	0.11
Jejunum	10	12	13	13	15	15	L<0.001	0.15	$y = 0.04x + 11$	0.4
Ileum	7	8	9	10	10	11	L<0.001	0.16	$y = 0.04x + 7$	0.3
Total small intestine	20	23	25	26	29	29	L<0.001	0.27	$y = 0.09x + 21$	0.6
Caeca	2.8	3.3	3.0	3.6	2.8	3.2	NS	-	-	0.14
Colon	0.71	0.71	0.73	0.71	0.78	0.82	NS	-	-	0.045

[1] Diet denominations indicate the percentage of pea starch in the starch fraction of the diet. L: linear regression; Q: quadratic regression; NS: Not significant; SEM: Pooled standard error of the mean; Number of replications = 6 pairs of cages.

Table 6.3. Effect of the proportion of dietary wheat and pea starch on digestive tract pH and organ weight of laying hens at 46 weeks of age

	Diets						Regression			SEM
	0[1]	20	40	60	80	100	*P* value	R^2	Equation	
pH										
Crop	5.06	5.05	5.12	4.93	4.99	4.79	L=0.033	0.04	y = -0.002x + 5.11	0.042
Gizzard	4.56	4.61	4.59	4.54	4.55	4.54	NS	-	-	0.027
Ileum	6.65	6.72	6.57	6.69	6.47	6.29	L=0.029	0.04	y = -0.003x + 6.74	0.054
Caeca	6.07	6.24	6.31	6.19	6.14	6.24	NS	-	-	0.033
Organ weight (g as-is basis)										
Heart	12	9	10	11	10	10	NS	-	-	0.3
Pancreas	2.8	3.0	3.2	3.2	3.5	3.3	L=0.002	0.09	y = 0.005x + 2.9	0.06
Liver	66	66	67	69	64	64	NS	-	-	1.1

[1] Diet denominations indicate the percentage of pea starch in the starch fraction of the diet.
L: linear regression; Q: quadratic regression; NS: Not significant; SEM: Pooled standard error of the mean; Number of replications = 6 pairs of cages.

Table 6.4. Effect of the proportion of dietary wheat and pea starch on h required to excrete 30% of the dietary marker in laying hens at 46 weeks of age

	Diets						Regression			SEM
	0[1]	20	40	60	80	100	*P* value	R^2	Equation	
Time (h)	4.6	5.7	5.8	4.8	5.7	5.0	NS	-	-	0.17

[1] Diet denominations indicate the percentage of pea starch in the starch fraction of the diet.
NS: Not significant; SEM: Pooled standard error of the mean; Number of replications = 6 pairs of cages.

Table 6.5. Effect of the proportion of dietary wheat and pea starch on GLP-1 and PYY serum concentrations (pg/mL) of laying hens at 46 weeks of age

	Diets						Regression		SEM
	0[1]	20	40	60	80	100	*P* value	R^2	Equation	
GLP-1	445	593	744	651	1008	748	L<0.001	0.17	$y = 4x + 510$	49.0
PYY	433	489	429	519	365	327	Q<0.001	0.28	$y = -0.04x^2 + 3x + 435$	13.3

[1] Diet denominations indicate the percentage of pea starch in the starch fraction of the diet.
L: linear regression; Q: quadratic regression; NS: Not significant; SEM: Pooled standard error of the mean; Number of replications = 12 hens.

increase in other components in the diets, which introduced the possibility that constituents such as fat and fibre could activate the ileal brake (Zhou et al., 2008; van Avesaat et al., 2015). Chemical analyses showed that diet fat concentrations were similar (2.3±0.4%; range 1.9-2.8%), suggesting a minimal effect. Dietary fibre concentrations increased from 21.5 for the 0% PS diet to 24.4% for the 100% PS diet, but the soluble fibre differences were small (range – 2.0 to 2.6%). Changes in fibre content have the potential to activate L-cells via their fermentation products (SCFA), with most fermentation associated with the soluble fibre fraction, so again the potential for L-cell activation appears small. Overall, diet analysis suggests that confounding effects were minor compared to the large changes in the nature of starch. Diet imbalance of other nutrients was likely not major as performance criteria presented in Chapter 4 show that laying hens met or exceeded Lohmann performance criteria (Lohmann Tierzucht, 2016).

Feeding laying hens diets with variable PS concentrations affected digestive tract weight, but the effect was not the same as seen for broiler chickens (Chapter 5). Whereas the size and content of most sections of the broiler digestive tract increased in a linear fashion with diet PS concentration, this was not always the case for laying hens in the present study. Crop empty weight was not affected by PS concentration despite a linear increase in feed intake (Chapter 4), while crop content weight changed quadratically with an estimated maximum content at 50% PS. As the gizzard regulates the flow of feed from the crop (Chaplin et al., 1992), the quadratic effect found in crop digesta content weight could be a direct effect of gizzard action. This idea is supported by the finding that gizzard weight was heavier in treatments with mid-concentrations of PS inclusion. It is also noteworthy that mid-concentrations of PS also resulted in maximum weight gain (Chapter 4), despite not coinciding with highest feed intake. Gizzard development is associated with improved digestibility (Hetland et al., 2003; Svihus et al., 2004) resulting from increased grinding capability and longer exposure of feed to proventriculus hydrochloric acid and digestive enzymes, and potentially because of gastroduodenal reflux (Svihus, 2011). There is no obvious reason for the gizzard weight response to diet PS, as pellet quality was similar for all treatments, and the linear increase in starch mixture content was the only difference in diet content. This suggests that the quadratic response was due to a physiological rather than a physical action. It is possible that the effect observed in gizzard function is associated with a corresponding quadratic effect of PS on PYY serum concentrations. Evidence in mammals shows that increasing plasma PYY concentrations are associated with a decrease in gastric

emptying through the activation of Y2 brain receptors (Savage et al., 1987; Chen et al., 1997). As higher concentrations of PYY were found in PS mid-ranges, this suggests that, like in mammals, PYY could produce a similar effect in chickens, reducing gastric emptying and improving digestibility. In addition to heavier body weights, hens fed the mid-range PS diets produced eggs with better eggshell quality (specific gravity; Chapter 4), which provides further support for this hypothesis. A more prolonged retention time in the gizzard could promote better calcium solubilization, which increases calcium availability for eggshell deposition (Guinotte et al., 1995). Also supportive of increased digestibility due to gizzard activity is the trend (P = 0.064) for a quadratic response in small intestine empty weight with a minimum at 34% PS. If gizzard activity, as indicated by weight, increased digestibility this could lead to a reduced requirement for small intestine development. This result is similar to that reported by Shynkaruk (2017) in broilers exposed the various dark period lengths, who found an inverse relationship between gizzard and small intestine empty weights.

The amount of digesta content increased linearly with PS inclusion level from the gizzard to the ileum, which is possibly the result of the direct effect found between PS concentration and feed intake (Chapter 4). In addition, both ileum empty weight and length, as well as cecal length, increased with PS concentration, providing support to the hypothesis of L-cells activation through the presence of glucose and fermentation products such as SCFA, resulting in glucagon-like peptide-2 release. This peptide is co-secreted along with GLP-1 from intestinal L-cells in response to carbohydrates such as glucose and fat, including SCFA, resulting in the promotion of intestinal growth (Tsai et al., 1997; Ghatei et al., 2001; Burrin et al., 2003). The presence of starch along the small intestine increases with dietary PS inclusion level (Chapter 5). Starch can be subjected to enzyme digestion, releasing glucose, or to bacterial fermentation, with the resulting production of SCFA.

All digestive tract pH values fell within the normal range for laying hens (Steenfeldt et al., 2007; Ruhnke et al., 2015). A negative linear relationship between dietary PS concentration and pH was found in the crop and ileum, suggesting increased fermentation with increasing PS concentration (Józefiak et al., 2008). In the case of the crop, changes in pH associated with PS starch concentration level are not due to substrate availability as the starch concentration was the same in all diets. It is possible that PS is fermented more readily than WS, but this is

counterintuitive based on their chicken digestibility characteristics. Therefore, it is more likely that pH changes are from increased fermentation due to the longer residence of feed in the crop (Shires et al., 1987; Józefiak et al., 2006). Regarding ileum pH, the presence of starch in the ileum increases with PS inclusion level according to research done in broiler chickens (Chapter 5), providing more substrate for bacterial fermentation.

Dietary PS concentration did not alter heart or liver size. However, pancreas weight increased linearly with the concentration of PS. Feed intake has been linked to pancreas size (Nitsan et al., 1974) and insulin secretion (Vilsbøll et al., 2003), and therefore, it can be an explanation for the observed increase in pancreas size. Alternatively, the increase in pancreas size could be the result of the activation of nutrient sensing mechanisms. GLP-1 has shown to have insulinotropic effects in mammals (Mojsov et al., 1987). Histological analysis has demonstrated the presence of GLP-1 receptors in the chicken pancreas as well, although an effect of chicken GLP-1 on insulin secretion has not been confirmed (Watanabe et al., 2014). As GLP-1 serum concentrations were found to increase linearly with PS, it is possible that the PS effects observed on egg production could be the result of an increment of pancreas size and insulin secretion in response to both feed intake and GLP-1, promoting protein accretion.

Dietary PS affected both GLP-1 and PYY serum concentrations, supporting the activation of L-cells (Nishimura et al., 2016; Aoki et al., 2017a). Meal size has been shown to positively affect the release of these neuropeptides (Monir et al., 2014a; Aoki et al., 2017a). As feed intake increased with PS concentration (Chapter 4), it is impossible to determine whether starch type, meal size or both affected GLP-1 concentration, which increased in a linear fashion with PS level. In a previous experiment conducted in broilers, SCFA analysis in the distal ileum showed that, although starch concentration increased with dietary PS, SCFA concentration followed a quadratic effect. In contrast to GLP-1, PYY showed a quadratic effect, with a maximum in the intermediate concentrations of PS. Thus, assuming a similar SCFA concentrations in the distal ileum of laying hens, this result suggests that PYY secretion could be more susceptible to the activation of L-cell SCFA receptors than glucose, as evidence suggests in mammals (Zhou et al., 2008; Singh et al., 2012; Psichas et al., 2015b; Brooks et al., 2017). As mentioned before, PYY concentration level could be related to the effects observed in the gizzard and crop contents, as

well as, body weight gain, eggshell quality and small intestine empty weight, by reducing gastric emptying.

An increase of intestinal transit time and a reduction of gastric emptying is observed in mammals with an increase in PYY (Savage et al., 1987; Ohtani et al., 1993; Chen et al., 1997). However, no effect of dietary PS was found on total digestive tract passage rate. A longer feed retention in the gizzard is associated to improved digestibility, and although passage rate shows indications of longer retention time in the proximal digestive tract in mid ranges of PS, increased digestibility could result in a faster passage rate along the small intestine, resulting in a lack of effect of PS concentration on total digestive tract passage rate (Svihus et al., 2002). Alternatively, in mammals gastro-inhibitory and excitatory effects of PYY are mediated by receptors Y2 and Y1 respectively, which are selectively activated depending on the PYY concentration levels (Chen et al., 1997). Current knowledge regarding the chicken NPY system remains rather limited, but characterization of chicken Y receptors have shown a certain degree of divergence when compared to mammals (Salaneck et al., 2000; Berglund et al., 2002; Holmberg et al., 2002; Lundell et al., 2002; Bromee et al., 2006). Consequently, the lack of effect on digestive tract passage rate found in laying hens as PYY concentration increased could be the result of NPY system differences between chickens and mammals, particularly in the small intestine and distal sections on the digestive tract.

The objectives of this study were to evaluate the effect of rate and extent of starch digestion on physiological and morphological changes in the laying hen digestive tract, with emphasis on activation of the ileal brake. It was hypothesized that as dietary PS increases, more carbohydrate fermentation would occur in the distal small intestine, which would be reflected by lower pH values. The presence of glucose and SCFA in the distal small intestine would also activate L-cells, increasing serum GLP-1 and PYY as well as decreasing passage rate. Our findings indicate that feeding increasing concentrations of PS affected digestive tract morphology and serum GLP-1 and PYY concentrations in a fashion that supports activation of L-cells. Although there are indications that passage rate in the proximal digestive tract might be reduced in a quadratic fashion following serum PYY concentrations, the anticipated change in overall passage rate was not found. These results suggest that avian species may respond in a different fashion to L-cell activation.

7.0 Assessing the effect of rate and extent of starch digestion on broiler and laying hen feeding behaviour

Activation of the ileal brake has shown to reduce feed intake in mammals, but despite other indications of ileal brake activation as a result of feeding PS in the current research, feed intake was not affected uniformly in broilers and laying hens. Feed intake decreased for male broilers, was not affected in female broilers and increased in laying hens as the dietary concentration of PS increased. How feed intake and ileal brake activation affect feeding behaviour is not known and therefore the objective of this chapter was to assess the effect of dietary pea starch concentration on broiler and laying hen feeding behaviour. These trials were run concurrently with the previous research presented in this thesis.

7.1 ABSTRACT

Feeding behaviour can be affected by diet composition as a result of diet density (bulkiness), the rate and extent of ingredient digestion, and activation of nutrient sensing mechanisms. It was hypothesized that the presence of starch in the distal small intestine would activate the ileal brake, increasing satiety, thereby changing feeding behaviour. Two semi-purified starch sources with differing *in vitro* digestibility, wheat (WS, rapidly digested), and pea (PS, slowly digested), were used in four diets containing equal amounts of starch, but differing in WS/PS ratios (100/0, 80/20, 60/40, 0/100). Diets were fed to Ross 308 male (944) and female (1056) broilers housed in 32 litter floor pens from 0-28 d. Similar diets were fed to 192 laying from 26-46 weeks of age, housed in 16 experimental units. Video recordings (24 h) were taken at 27-28d from 4 individually marked broilers per pen, and at 46 weeks of age from 2 hens per experimental unit. Focal observations were used to record the initiation and end of every feeding (F) and drinking bout (D, only on broilers) over 24 h to calculate number of bouts (N), bout length (BL), time between bouts (TBB), total time (TT) during the photo and scotoperiods, and time until first night bout (T1N). Data were analyzed as a 4 (diet) x 2 (gender) factorial arrangement (broiler) or one-way ANOVA (laying hen) using the SAS 9.4 GLIMMIX procedure. Significance was considered when $P<0.05$. No interactions or night feeding were found. Diet affected broiler NF, TBFB and TFT, but changes appeared random and did not support a satiety hypothesis. Males had more NF and TFT than females, but shorter FBL, suggesting increased hunger. Drinking behaviour was not affected, except for longer TBDB for females. No effect of diet on day-time hen feeding behaviour was found, but night NF increased, and T1NF decreased as PS increased suggesting increased hunger with PS concentration. In summary, no effect of dietary PS on broiler feeding behaviour was found. However, hen night feeding behaviour suggests that PS increased hunger, in contrast to the hypothesis.

7.2 INTRODUCTION

Feeding behaviour is affected by a variety of factors including diet. For example, concentration of nutrients or the inclusion of non-starch polysaccharides (NSP) can affect feeding behaviour. In an experiment, energy level and the addition of NSP were assessed on

laying hens performance and feeding behaviour (van Krimpen et al., 2009). Although an interaction of the main factors was found during the rearing period, the inclusion of NSP in diets that provided 2630 kcal/kg, resulted in higher feed intake and more grams of diet consumed by minute, but it reduced total feeding time compared to diets with the same energy level but no NSP. This difference disappeared when the authors fed diluted diets. Similarly, rate and extent of starch digestion also affect feeding behaviour. In another experiment, isoenergetic diets with or without resistant starch were fed to pigs (Souza da Silva et al., 2014). Resistant starch resulted in lower digestibility, but it did not affect overall feed intake. However, pigs fed resistant starch diets had longer meals, a longer time between meals and fewer meals per day.

Feeding behaviour is controlled by hypothalamus activation of hunger and satiety mechanisms in response to nutrient composition of the diets and body energy storage status (Richards and Proszkowiec-Weglarz, 2007). Nutrient sensing mechanisms in the digestive tract result in the release of a number of hormones and neuropeptides that convey this information to the hypothalamus and the rest of the body through paracrine and endocrine routes (Honda et al., 2017), and result in the modulation of feed intake and meal patterns, as well as other physiological changes. Research has shown that the partial to total replacement of semi-purified wheat starch (WS) with semi-purified pea starch (PS), which is digested more slowly and to a lesser extent, in poultry diets results in a number of changes in parameters related to the digestive tract (Chapters 5 and 6). In general, a positive relationship between PS concentration and the relative weight of digestive content was found in both broilers and laying hens (Chapters 5 and 6). However, other effects of PS differed between broilers and laying hens. For instance, while empty digestive tract weight and length in broilers increased with PS concentration, laying hen digestive tract weight and length decreased for low to mid-range concentrations of PS and then increased when PS inclusion concentration surpassed 50%. Other differences were found in the venous concentrations of glucagon-like peptide 1 (GLP-1) and peptide tyrosine-tyrosine (PYY), two hormones that are released in response to nutrient sensing (Dumoulin et al., 1998). These hormones were not affected by PS concentration in broilers. In contrast, concentration of dietary PS had a positive effect on laying hen venous GLP-1 concentration while PYY responded to PS in a quadratic fashion. These results suggest broilers and laying hens respond differently to the rate and extent of starch digestion.

Extensive genetic selection has been applied to both broilers and laying hens to increase growth rate and egg production, respectively. This pressure has resulted in high feed intake in broilers, approaching full gastrointestinal capacity, and a growth rate that allows them the reach and surpass an adult laying hen weight in approximately five weeks. Although body weight might be similar, developmental status, size of the digestive tract and composition of the microbiota are very different between a four week-old broiler and a fully developed laying hen (Bedford, 1996), which in addition to divergent selection pressures explain why their physiological and behavioural responses can differ when exposed to the same treatment.

Both the use of more slowly digestive starch and elevated GLP-1 and PYY concentrations have been associated with increased satiety (van Avesaat et al., 2015). Significant changes in satiety are usually accompanied by changes on feed intake. However, subtle effects can be assessed by observing feeding behaviour (Nielsen, 1999). For instance, satiety is associated with a longer time between feeding bouts (Tolkamp et al., 2011). Thus, the objective of this study was to assess the effect of slowly or poorly digestive starch inclusion in chicken diets on feeding behaviour of broilers and laying hens.

7.3 MATERIALS AND METHODS

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (Canadian Council on Animal Care, 1993, 2009).

Experiments described here belong to larger studies that included performance (Chapters 3 and 4), and physiological and morphological measures (Chapters 5 and 6). Out of six experimental treatments, four were chosen for behavioural observations based on performance effects reported in the literature under the application of treatments based on the rate and extent of starch digestion (Weurding et al., 2003; Gutierrez del Alamo et al., 2009).

7.3.1 *Experimental diets*

In both broiler and laying hen experiments, diets were formulated to be identical (Table 7.1) with the exception of the starch source. Semi-purified WS (Archer Daniels Midland

Table 7.1. Ingredient and calculated composition of treatment diets

Ingredient (%)	Broiler trial			Laying hen trial
	Starter (0.4 kg/bird)	Grower (1.4 kg/bird)	Finisher	
Semi-purified starch	47.49	53.66	59.35	53.42
Soybean meal	39.08	32.65	27.31	26.30
Porcine meal	5.00	5.00	5.00	5.00
Oat hulls	2.00	2.00	2.00	2.00
Soybean oil	2.71	3.50	3.36	1.51
Monocalcium phosphate	0.97	0.78	0.68	0.70
Limestone	1.19	0.92	0.86	9.65
Sodium chloride	0.37	0.37	0.37	0.34
Vitamin/mineral premix[1,2]	0.50	0.50	0.50	0.50
Choline Chloride	0.10	0.10	0.10	0.10
DL-Methionine	0.52	0.46	0.42	0.43
L-Threonine	0.07	0.06	0.05	0.05
Calculated composition (%)				
AME (kcal/kg)	3,025	3,150	3,200	2,800
Dry matter	88.61	87.99	88.22	89.79
Crude protein	25.09	22.43	20.24	19.35
D-Lysine	1.54	1.37	1.23	1.17
D-Methionine	0.70	0.63	0.58	0.58
D-Methionine-Cysteine	0.94	0.84	0.76	0.75
D-Threonine	0.83	0.73	0.65	0.62
Crude fat	4.34	5.14	5.01	3.09
Calcium	1.05	0.90	0.85	4.10
Available phosphorus	0.50	0.45	0.42	0.42

[1]Broiler: supplied per kilogram of diet: vitamin A (retinyl acetate + retinyl palmitate), 11000 IU; vitamin D3, 2200 IU; vitamin E (dl-α-tocopheryl acetate), 300 IU; menadione, 2.0 mg; thiamine, 1.5 mg; riboflavin, 6.0 mg; niacin, 60 mg; pyridoxine, 4 mg; vitamin B12, 0.02 mg; pantothenic acid, 10.0 mg; folic acid, 0.6 mg; biotin, 0.15 mg; copper, 10 mg; iron, 80 mg; manganese, 80 mg; iodine, 0.8 mg; zinc, 80 mg; selenium, 0.3 mg; calcium carbonate, 500 mg; antioxidant, 0.625 mg; wheat midds, 3772.73 mg.

[2]Laying hen: supplied per kilogram of diet: vitamin A (retinyl acetate + retinyl palmitate), 8000 IU; vitamin d3, 3000 IU; vitamin/e E (dl-α-tocopheryl acetate), 25 IU; menadione, 1.5 mg; thiamine, 1.5 mg; riboflavin, 5.0 mg; niacin, 30 mg; pyridoxine, 1.5 mg; vitamin B12, 0.012 mg; pantothenic acid, 8.0 mg; folic acid, 0.5 mg; biotin, 0.06 mg; copper, 5 mg; iron, 80 mg; manganese, 80 mg; iodine, 0.8 mg; zinc, 80 mg; selenium, 0.3 mg; calcium carbonate, 500 mg; ethoxyquin, 0.625 mg; wheat middlings, 3822.79 mg.

Company, Montreal, QC, Canada) and PS (Parrheim Foods, Saskatoon, SK, Canada) were used after confirming via *in vitro* analysis that WS was digested more rapidly than PS (Chapter 3). The purity of the starch sources was not equal (wheat = 91% *vs*. pea = 80% starch), and therefore purified pea protein (Parrheim Foods, Saskatoon, SK, Canada) was added to the WS to equalise starch, protein and fibre concentrations. The starch fraction of the diets contained 87% WS and 13% pea protein in 0% PS diet. Thereafter, the appropriate amount (20, 40 or 100%) of the WS-pea protein mixture was replaced by PS to produce the remaining treatments. Because of the fine nature of starch sources, all diets were pelleted, and processing temperature did not surpass 85°C. Diets were manufactured at the Canadian Feed Research Centre (CFRC, North Battleford, SK, Canada). An inverse relationship between starch digestibility and dietary PS inclusion concentration was confirmed through *in vivo* assessment in broilers at 28 d of age (Chapter 5).

7.3.2 *Birds and bird housing*

Broiler trial. A total of 2,000 Ross 308 broiler chicks were obtained from a commercial hatchery (Sofina Inc., Wynyard, SK, Canada) and allocated randomly by gender to 12 pens, in each of four rooms, at the Poultry Centre at the University of Saskatchewan (four replications per diet x gender treatment). The experiment was a randomized complete block design with four diet by two gender treatments in each room; the room was assigned as the blocking unit.

Birds were raised under controlled conditions of light (23L:1D, 30 lux during the first 2 d followed by 16L:8D, 10 lux until the end of the experiment) and temperature (starting at 33°C and reduced daily to reach 21°C by d 25). Feed and water were offered *ad libitum*. Wheat straw was used as bedding material in floor pens (4.6 m^2). Each pen was furnished with a tube feeder (36 cm diameter from 0-21 d and 43 cm diameter thereafter) and six nipple drinkers. Based on 32 d weights obtained from the Ross Performance objectives (Aviagen, 2014), 66 female or 59 male chicks were allocated to each pen, to achieve a final estimated bird density of 31 kg/m^2.

Laying hen trial. At the age of 16 weeks, Lohmann LSL pullets were randomly allocated in groups of six into 32 Layer Specht conventional cages (503 cm^2/bird, 60 cm feeder trough, one lubing nipple drinker). Cages were located in the same tier, and two adjacent cages were considered an experimental unit, resulting in four replications of 12 hens per treatment diet, arranged in the completely randomized design. The barn was maintained at approximately 21^oC,

and birds were given 14 h of light (10lux) per d (with dusk and dawn periods of 15 min each); feed and water were provided *ad libitum*. Birds were fed age-appropriate commercial diets until the trial started at 26 wk of age. The trial lasted 20 wk.

7.3.3 *Data collection*

Behavioural video recordings were planned to coincide with physiological and morphological digestive tract data collections.

Broiler trial. On d 27 or 28 four randomly selected males or females per pen were individually marked along the back using carbon-based ink to enable focal determination of feeding and drinking behaviour. An infrared video camera system (WV-CF224FX; Panasonic Corporation of North America, Secaucus, NJ) was mounted along the ceiling above each male and female pen to capture the entire pen area, and activity was recorded in a continuous real-time mode. Broiler male pens were recorded at 27 d of age, while female recording were captured the following day.

At a later date, recorded video was observed using Genetec Omnicast Software (Genetec Inc., Montreal, Quebec, Canada) and time to specific activities, such as initiation and termination of feeding for each individual marked focal bird was recorded. The behaviour of individual birds was averaged per pen, and each pen was considered the experimental unit. Feeding behaviour was defined as a marked bird with its head within the rim of the feeder and oriented downward. Drinking behaviour was defined as a marked bird standing under the drinker with its head orientated upward. Feeding or drinking events with 10 or fewer seconds between them were considered the same bout (Bokkers and Koene, 2003). No night feeding or drinking were observed, and thus data collection included the number, duration and time between feeding or drinking bouts, as well as total feeding or drinking time during the photoperiod (16L). Although data were carefully collected, the authors realize that these feeding and drinking definitions could include other activities (e.g. playing with feed; object pecking), as actual feed and water consumption were not measured. Any use of the words feeding and drinking in this manuscript will be according to the definitions previously stated.

Laying hen trial. At 46 wk of age, one randomly selected hen per cage (two hens per experimental unit) was individually marked using carbon-based ink to perform behaviour

recordings. An infrared video camera system (WV-CF224FX; Panasonic Corporation of North America, Secaucus, NJ) was mounted in front of each experimental unit using a tripod to capture the entire area in a 24 h continuous recording real-time mode.

At a later date, Genetec Omnicast Software (Genetec Inc., Montreal, Quebec, Canada) was used to record the time of specific activities for behavioural analysis. Following the same method as used in the broiler pens, the behaviour of individual birds per experimental unit was averaged. Feeding behaviour was defined as a marked bird with its head in the feeder. Due to the difficulty in observing the back of the cage, drinking behaviour was not included in this trial. Feeding events with 10 or fewer seconds between them were considered the same bout. Data collection included the number, duration and time between feeding bouts, as well as total feeding during the photo (14L) and the scotoperiods (10D). In addition, the time between the end of the dusk period and the first night feeding bout was recorded.

7.3.4 *Dietary chemical analyses*

Diets were analyzed for total starch (TS), moisture, fat, crude protein (CP), ash, and dietary fibre (DF) following AOAC standard methods (AOAC, 2006). Starch was determined using the Megazyme Total Starch Kit (Megazyme Inc., Chicago, IL, USA) following the 996.11 AOAC method. Moisture was measured following method No. 930.15. Fat content was determined by ethyl ether extraction using Goldfish Extraction Apparatus (Labconco model 35001; Labconco, Kansas, MO, USA) following AOAC method 920.39. Crude protein of the diets was determined using a Leco N analyzer (Leco FP-528; Leco Corp., St. Joseph, MI, USA) following 990.03 method and a 6.25 multiplication factor was used to convert N to CP. Ash was measured (method 942.05) using a muffle oven (Lindberg/Blue BF51842, Asheville, NC 28804, USA). Dietary fibre was determined using Megazyme Total Dietary Fibre kit (Megazyme Inc., Chicago, IL) following the AOAC 985.29 method.

7.3.5 *Statistical analyses*

Before statistical analysis behavioural data was validated by a second observer blind to treatment. Normality of the residuals and homogeneity of variance was checked in all collected data previous to statistical analysis. Data was transformed when required to meet the assumptions. Broiler data were analyzed following a randomized complete block design, using

the room as blocking factor, diet and gender as the main effects, and pen as the experimental unit. Room effects were not significant ($P > 0.05$) and therefore were removed from the analyses, allowing analysis as a completely randomized design. Data were analyzed using ANOVA (GLIMMIX model procedure) with SAS 9.4 (SAS Institute, 2004) after testing the compliance of ANOVA assumptions. The number of broiler visits to the feeder was reciprocally transformed (1/N) to meet the assumptions. Laying hen day and night feeding data were analyzed each with ANOVA (GLIMMIX model procedure) using diet as the main effect. After assumption testing of the data, total night feeding required to be square root transformed to meet statistical assumptions. In all cases, when significant differences were found in the ANOVA analyses, Tukey's test was applied for mean separation. All differences were considered at $P \leq 0.05$ and trends were considered when $0.10 \geq P > 0.05$.

7.4 RESULTS

Performance and physiological results from this research are reported in Chapters 3, 4, 5 and 6. For the most part, performance criteria met or exceeded the expectation of the primary breeders (Aviagen, 2014; Lohmann Tierzucht, 2016). Diets were formulated based on the analysis of samples derived from the starch sources. However, the starch concentration in the PS used in final feed manufacture was lower than expected (61 *vs*. 80%). As a result of this, starch concentration decreased with increasing PS inclusion for all diets (e.g. broiler finisher: 6% reduction from 0% PS to 100% PS diet), with a slight increase of all other components of the diets. Nevertheless, PS concentration effects were found in a number of variables regardless of confounding effects of diet (Chapters 3, 4, 5 and 6). Analyzed nutrient values were similar to the calculated values presented in Table 7.1.

7.4.1 *Broiler behaviour*

Individual feeding behaviour was highly variable. For example, most time feeding was spent by a focal male which fed for 2 h, 44 min and 22 s, while the least time feeding by a focal male was only 33 min and 38 s. General observations of focal broiler daily feeding behaviour patterns were very similar between diets with no feeding events during the scotoperiod, followed by compensatory feeding during the first 2 h of the photoperiod. The remaining feeding

behaviour was distributed along the photoperiod, with a slight increase at the end of the photoperiod (Figure 7.1).

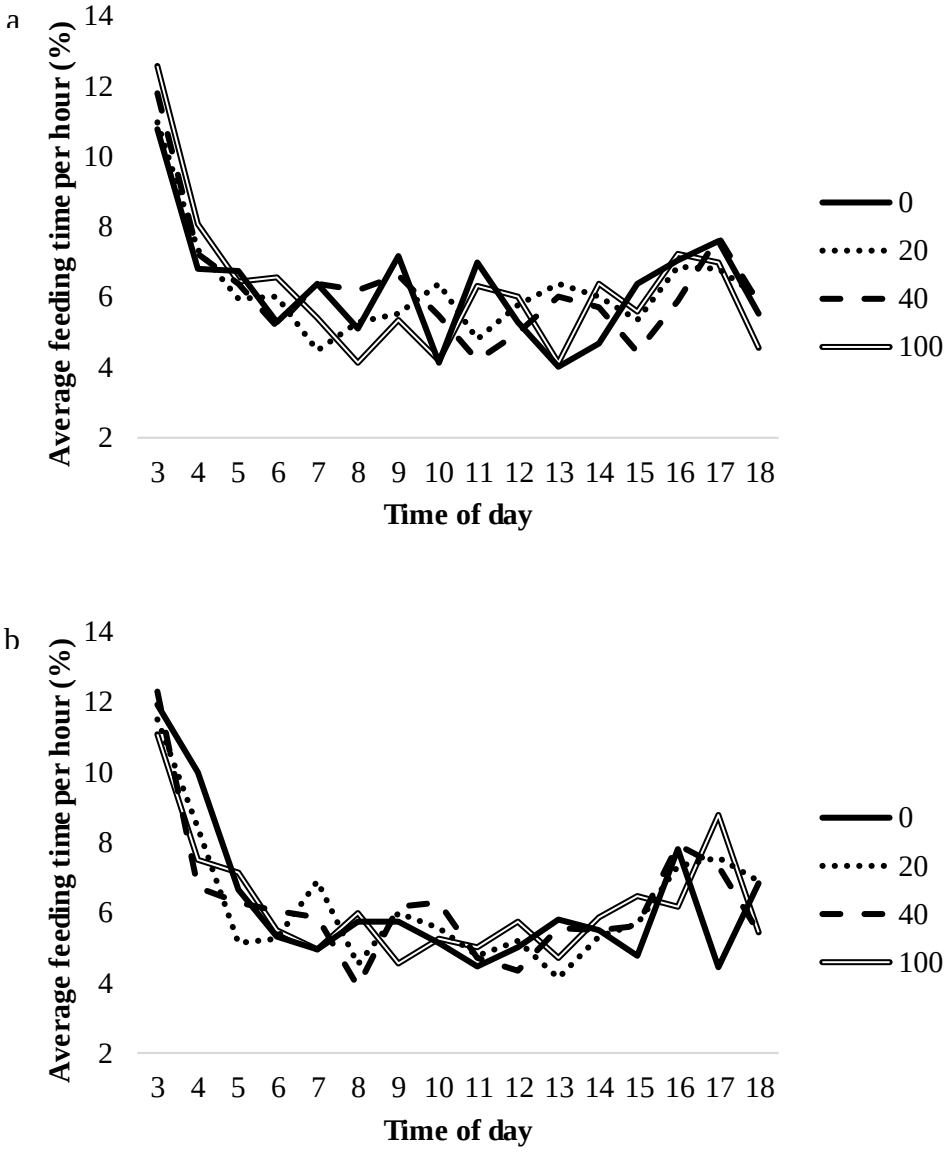

Figure 7.1. Average feeding time per h in (a) female, and (b) male broilers fed diets differing in starch fraction composition, from 100% semi-purified wheat starch (0) to 100% semi-purified pea starch (100). Diets denominations indicate the percentage of pea starch in the starch fraction of the diet. Photoperiod started at 3 am and ended at 7 pm

None of the focal broilers fed during the dark period and as a consequence data presented here corresponds to the photoperiod. Broilers visited the feeder on average between 64 and 85 times per day and spent on average a total of 78 min (4,680 s) at the feeder daily (Table 7.2).

Table 7.2. Effect of proportion of dietary wheat and pea starch on broiler 24h feeding behaviour (27-28 d of age)

	Diet (D; %PS)					Gender (G)			DxG	SEM
	0	**20**	**40**	**100**	***P* value**	**F**	**M**	***P* value**		
N	80^{ab}	68^{ab}	85^{a}	64^{b}	0.034	50	98	<0.001	NS	5.3
TBFB (s)	797^{ab}	896^{a}	747^{b}	912^{a}	0.031	1093	583	<0.001	NS	51.0
TFT (s)	4650^{ab}	4223^{b}	5361^{a}	4414^{ab}	0.028	3893	4085	<0.001	NS	199.2
FBL (s)	68	67	71	75	NS	80	39	<0.001	NS	3.0

N: number of visits; TBFB: time between feeding bouts; TFT: total feeding time; FBL: feeding bout length.

$^{a, b}$ Means with common letters do not differ significantly within each main treatment group (diet, and gender).

P value: indicates ANOVA *P* value; F: female; M: male; SEM: Pooled standard error of the mean. Number of replications = 4.

Diet affected broiler feeding behaviour at 27-28 d of age. Broilers fed 40% PS diets visited the feeder more than those fed 100% PS and values for birds from the 0 and 20% PS treatments were intermediate and not different from the 40 and 100% PS values. Time between feeding bouts, which did not account for the period between the last feeding bout before darkness and the first feeding bout after darkness, was higher for the 20 and 100% PS treatments in comparison to the 40% treatment, while the mean value for the 0% PS treatment was intermediate and did not differ from the other treatments. Birds on 40% PS treatment had more total feeding time than those in the 20% treatment, with other treatments being intermediate and not different from any of the treatments. Feeding bout length was not affected by dietary treatment. Males visited feeders more, more frequently, had shorter feeding bouts and spent more total time at the feeder than females.

Broilers visited the drinker between 82 and 103 times during the day and spent on average 86 min (5,160 s) daily at the drinker (Table 7.3). Diet did not affect any of the variables regarding broiler drinking behaviour. Gender only affected the frequency of visits to the drinker, with males spending shorter amounts of time between drinker visits than females.

Table 7.3. Effect of proportion of dietary wheat and pea starch on broiler 24 h drinking behaviour (27-28 d of age)

	Diet (D; %PS)					Gender (G)				
	0	**20**	**40**	**100**	***P* value**	**F**	**M**	***P* value**	**D x G**	**SEM**
N	95	85	103	82	NS	85	98	NS	NS	4.8
TBDB (s)	603	714	606	680	NS	718	583	0.046	NS	33.1
TDT (s)	5120	4844	5745	4996	NS	4922	5430	NS	NS	165.5
DBL (s)	59	62	60	65	NS	63	60	NS	NS	2.3

N: number of visits; TBDB: time between drinking bouts; TDT: total drinking time; DBL: drinking bout length.
P value: indicates ANOVA *P* value; F: female; M: male; SEM: Pooled standard error of the mean. Number of replications = 4.

7.4.2 *Laying hen behaviour*

Individual feeding behaviour varied substantially between hens. Most time feeding by a focal hen was 5 h, 30 min and 39 s, while the least total time feeding was 17 min and 35 s. Daily feeding pattern was similar between diets. Scotoperiod was followed by compensatory feeding during the first hour decreasing to reach a minimum after approximately 5 h. Feeding time increased after, until anticipatory feeding occurred at the end of the photoperiod (Figure 7.2).

Laying hens visited the feeder between 157 and 188 times during the day, spending an average total time of 138 min (8,280 s) daily at the feeder (Table 7.4). No dietary effect on laying hen day feeding behaviour was found.

Night laying hen feeding behaviour differed markedly among diets (Table 7.5). Out of all focal birds observed 75% fed at night, but only 56% fed before 9 h of darkness (0% PS = 2/8; 20% = 4/8; 40% = 4/8; and 100% = 6/8 focal birds). The number of visits increased with PS inclusion, with hens fed the 100% PS diet visiting the feeder almost 3 times more than those fed the 0% PS diet. Additionally, the time to the first night feeding event decreased as PS concentration increased. Laying hens on the 0% PS diet did not approach the feeder until on average more than 7.5 h (27398 s) of darkness had elapsed while those on 100% PS initiated feeding approximately 4 h (14609 s) after the lights went out. Total night feeding time only showed a trend ($P = 0.100$) to differ between treatments.

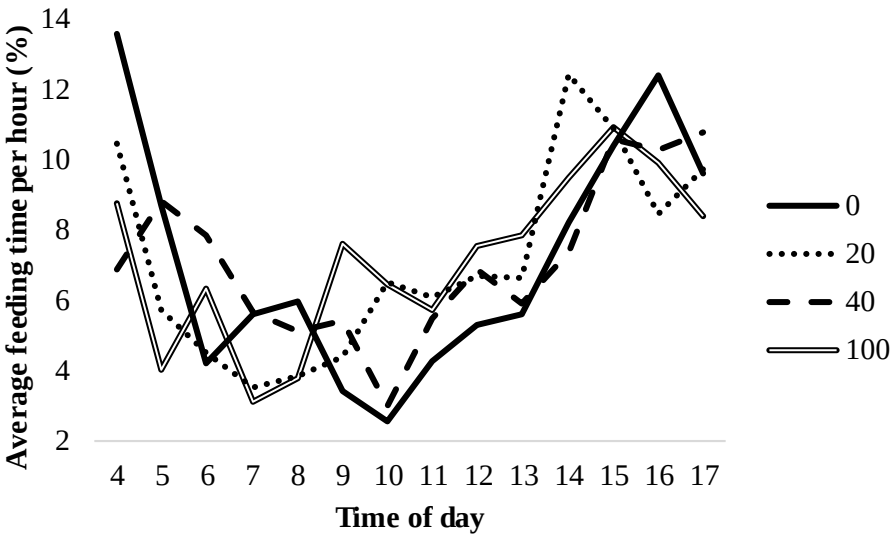

Figure 7.2. Average feeding time per h in laying hens fed diets differing in starch fraction composition, from 100% semi-purified wheat starch (0) to 100% semi-purified pea starch (100). Diets denominations indicate the percentage of pea starch in the starch fraction of the diet. Photoperiod started at 4 am and ended at 6 pm

Table 7.4. Effect of the proportion of dietary wheat and pea starch on laying hen day feeding behaviour (46 weeks of age)

	Diets (%PS)				*P* value	SEM
	0	**20**	**40**	**100**		
N	188	159	157	177	NS	6.1
TBFB (s)	264	302	276	259	NS	11.7
TFT (s)	8356	7353	8341	9300	NS	647.9
FBL (s)	42	44	53	50	NS	2.9

N: number of visits; TBFB: time between feeding bouts; TFT: total feeding time; FBL: feeding bout length
P value: indicates ANOVA *P* value; SEM: Pooled standard error of the mean. Number of replications = 4.

Table 7.5. Effect of the proportion of dietary wheat and pea starch on laying hen feeding behaviour during the scotophase (46 weeks of age)

	Diets (%PS)				*P* value	SEM
	0	**20**	**40**	**100**		
N	2.4[b]	3.3[ab]	3.8[ab]	9.4[a]	0.029	0.99
TBNFB (s)	703	2455	3072	3180	NS	530.9
T1NFB (s)	27398[a]	25586[b]	22059[c]	14609[d]	<0.001	2658.9
TNFT (s)	105	110	143	473	NS	60.8

N: number of night feeder visits; TBNFB: time between night feeding bouts; T1NFB: time to first night feeding bout; TNFT: total night feeding bout.
[a - d] Means with common letters do not differ significantly within each main treatment group (diet).
P value: indicates ANOVA *P* value; SEM: Pooled standard error of the mean. Number of replications = 4

7.5 DISCUSSION AND CONCLUSIONS

Differences in the definition of meal, in addition to experimental setup, feed type and formulation, observation length and accuracy among other characteristics make comparisons between behaviour studies in the literature virtually impossible (Nielsen et al., 2016). Nevertheless, the results for broiler feeding behaviour are very similar to those reported by Weeks et al. (2000), where data were collected using similar methodology, but at an older age. Laying hens have been reported to spend a higher proportion of time feeding than our findings indicate (17 *vs*. 22-37%, e.g. Mench et al., 1986; Tanaka and Hurnik, 1992; Webster, 2000). Although these observations were all made based on conventionally housed hens, other aspects of the experimental design and methodology were different (e.g. 24 h focal *vs*. scan observations of a period of time, age, genotype, diet, photoperiod).

In the production component of this work, no effect of the proportion of WS and PS was found on broiler feed intake (Chapter 3), however previous research has concluded that subtle increases in satiety can be correlated to a longer time between feeding bouts (Tolkamp et al., 2011). Our results indicate that feeding behaviour differs between treatments, but starch type did not appear to cause a consistent effect across PS concentration levels. As a result, these

differences do not support the hypothesis that PS increases satiety through a nutrient-sensing mechanism such as L cell activation.

One of the mechanisms hypothesized to be involved in improving feed efficiency when feeding slowly digestive or resistant starch is the ileal brake. According to this theory, PYY and GLP-1 are released from intestinal L-cells, causing an increase in feed transit time and enhance diet digestibility (Ferguson et al., 2000; Keenan et al., 2006; Zhou et al., 2008). The ileal brake is also associated with a reduction of feed intake and an increase in satiety (Alviña and Araya, 2004; Hasek et al., 2018). In the physiological component of this work, PS inclusion resulted in a number of changes in the digestive tract of broilers, including an increased amount of digestive contents in the crop, suggesting a reduced feed transit time (Chapter 5). However, we found that PS did not consistently affect feed intake or peripheral blood concentrations of GLP-1 and PYY in broilers (Chapter 5). In the same study it was found that proglucagon, GLP-1 precursor (Richards and McMurtry, 2009) gene expression was affected by the relative concentration of PS in the diet, but contrary to the hypothesis previously mentioned, proglucagon expression decreased as PS concentration increased (Chapter 5). Thus, both ELISA and gene expression results regarding neuropeptides GLP-1 and PYY in broilers suggested that the ileal brake was not being activated. Feeding behaviour supports these results by showing no effect of starch type on satiety. Likewise, no effect of diet on drinking behaviour was found either. Therefore, differences found in feeding behaviour between treatments were likely caused by high variability of the data and not a biological effect of the dietary treatments.

Males had a higher feed intake and final body weight than females (Chapter 3), which was accompanied by more visits and longer total time at the feeder, while bout length and intervals between bouts were shorter than for females. Similar trends were found by Shynkaruk (2017) who reported more male visits to the feeder, a trend for increased total time feeding and shorter intervals between bouts than in females. She did not find feeding bout length differences between males and females. These results suggest that the higher growth rate demands experienced by male broiler chickens increase hunger, resulting in more frequent visits to the feeder (Tolkamp et al., 2011). Higher levels of testosterone have been associated with shorter, more frequent feeding bouts in mice (Petersen, 1978), which might also explain gender differences found in broiler feeding behaviour.

Laying hen feed intake increased linearly with PS concentration (Chapter 4), however, no effect of diet was found on day-time feeding behaviour. The lack of effect suggests that hens fed lower PS diets might exhibit less feeding motivation and/or spend time performing other activities at the feeder when compared with those fed higher proportions of PS. Without measuring actual feed intake per feeding bout, it is impossible to determine whether a hen is feeding or spending time doing other activities (e.g. playing or object pecking; Bubier, 1996; Weeks and Nicol, 2006). Conventional cages have been reported to reduce the diversity of behaviours performed due to the lack of opportunities (Mench et al., 1986; Freire and Cowling, 2013). As a consequence, more time might be spent at the feeder in compensation.

While no night feeding was observed in focal broilers, laying hens spent a considerable amount of the dark period feeding. A more extended photoperiod, younger age and a larger housing area in broilers might increase their sleep requirements when compared to conventional caged hens. At the same time, less space and an increased number of disruptions during the night may have woken hens up more frequently. In addition, an effect of starch type was found on laying hen night feeding behaviour. The length of time hens spent without feeding shortened as the amount of PS increased. Anticipatory feeding, as judged by feeding behaviour, was similar among dietary treatments, suggesting that all birds initiated the scotoperiod with similar crop and digestive tract fill. This indicates that the PS effect was opposite to the expected increased satiety as PS increased as a result of activation of the ileal brake. These results are consistent with the positive relationship between PS concentration and laying hen feed intake (Chapter 4) and small intestine content (Chapter 6) previously found, implying that hunger increased with PS concentration. A higher degree of hunger, as indicated by earlier and more night feeding, might be the result of a combination of increased metabolic demand due to increased egg production (Chapter 4) and the lower than expected starch content in the diets.

In conclusion, broiler and laying hen feeding behaviour responded in a different manner to dietary PS. Physiological changes produced by dietary PS in broilers are not associated with changes in feeding behaviour. On the other hand, the positive linear association observed between egg production and PS concentration in laying hens may be related with an increase in hunger level reflected by a shortening of the time hens are able to spend without feed during the dark phase.

8.0 General discussion

8.1 Introduction and objectives

The global human population increased at a rate of 1.1% annually between 2013 and 2017 (Food and Agriculture Organization of the United Nations) and is expected to continue to increase in future decades (http://www.worldometers.info/world-population/). Corresponding to the increase in population is the need for increased food production. The poultry industry will play an important role in supplying the increased food requirement and global poultry numbers have increased in recent years and will continue to increase in the future (Mottet and Tempio, 2017). As a consequence of these changes, the poultry industry needs to continuously evolve to produce its products more efficiently.

In 2003, a study reported that the use of pea-corn instead of tapioca-corn, the former being more rapidly digested, in feed formulation improved broiler feed efficiency (Weurding et al., 2003). A similar result was reported some years later by Gutierrez del Alamo et al. (2009), using a variety of wheat sources with different digestibility coefficients, and in some cases the inclusion of pea to slow digestion rate even further. However, in this case, the effect of digestion rate on feed efficiency was quadratic, showing that there is a threshold on how much slowly starch digestion rate is required to obtain the desired effect. Thus, although the inclusion of starch sources that are more slowly digested seems to be a potential tool to improve feed efficiency, a number of questions arise, such as: Is it the nature of the starch, or the effect of other components of the grain that affect feed efficiency in broilers? Do laying hens respond in a similar manner to broilers with regards to the rate and extent of starch digestion? What are the mechanisms behind the positive effect on feed efficiency?

Chickens are very efficient at digesting starch, and all or most of the starch included in traditional diets (e.g. wheat, corn) is digested and absorbed prior to or in the jejunum. When more slowly digested starch is used, a higher proportion of starch reaches the ileum, and thus it is not only digested and absorbed at a slower rate, but it also alters the ileum digesta composition, affecting the energy sources available for ileum enterocytes, and the local microbiota and nutrient sensing mechanisms. The rate of absorption will affect the length of blood glucose availability and the intensity of the insulin response to regulate blood sugar concentrations. In addition, by reaching the ileum, glucose from starch digestion would be available for the ileum

enterocyte metabolism, reducing the need for other energy sources such as circulating blood glucose or glutamate. Even before its absorption, taste receptors present in enteroendocrine cells, such as L-cells, could activate the release of GLP-1 and PYY. These two hormones, involved in the ileal brake, decrease gastric emptying and promote insulin release and satiety in response to carbohydrate and fat. Finally, the starch present in the ileum could act as a prebiotic, providing a fermentation source for the local microbiota. This process would result in the production of short-chain fatty acids (SCFA), which in turn can modulate the local microbiota and activate nutrient sensing mechanisms as well.

In summary, a more slowly digestive starch could affect feed efficiency via a number of mechanisms, and at this point, it is unclear if any or all of them are playing a role. In order to assess some of these points, the results described by Weurding et al. (2003) and Gutierrez del Alamo et al. (2009) need to be confirmed using semi-purified starch sources, and without other potential confounding components associated with specific grains. The general objective of this study was to determine the effects of rate and extent of starch digestibility on the performance, physiology and behaviour of broiler chickens and laying hens using semi-purified starch sources. The specific hypotheses were that the inclusion of slowly or poorly digested starch in broiler and laying hen diets will: result in a more feed efficient production; increase the presence of starch in the distal digestive tract and as a consequence increase fermentation (lower pH and increased SCFA); and extend nutrient availability and lower pH values in the digestive tract of broilers under feed withdrawal conditions. Also, slowly or poorly digested starch will: activate the ileal brake; increase digesta content in the proximal digestive tract; increase blood concentrations and gene expression of GLP-1 and PYY; and activate satiety mechanisms and therefore alter feeding behaviour.

8.2 Diets

Diets were formulated to be equal except for the composition of the starch fraction. A diet base for all diets was made, to be later separated into six portions to make each diet for all six treatments. This was an effective way to approach diet manufacturing as it reduced the chance for differences between diets due to fractions other than starch. The use of semi-purified starch sources, although resulting in increased digestibility values when compared to complete sources, lessened potential confounding effects from grain components other than starch such as

saponins, tannins and fibre. Likewise, pea protein addition to the WS approximately equalized the protein, fat and fibre concentrations of the two starch sources. Overall, the formulation and manufacturing approach diminished differences in fat or fibre components in the diets that could have activated the ileal brake and made interpretation of the data more difficult.

Despite considerable effort to accurately formulate diets, variability in the starch content of the PS sample used in feed manufacturing clouded the interpretation of experimental data. Nevertheless, production results found in broilers support previous findings reported by Weurding et al. (2003), showing a positive effect of a starch source with a slower digestion rate on feed efficiency. Additionally, digestion rate had a quadratic effect on feed efficiency, supporting the results of Gutierrez del Alamo et al. (2009), who reported that as digestion rate was reduced, the initial positive response observed on feed efficiency disappeared. Similarly, the effect of PS found on energy partitioning had been previously reported by (Deep, 2018), who fed wheat- or pea-based diets to broiler breeder pullets and found a reduction of the expression of liver lipogenic enzymes with pea, possibly explaining the reduction in broiler body-fat reported in this thesis as PS concentration increased.

8.3 Performance

Despite differences in the bird type, the age of birds and length of exposure to the treatment diets, performance results indicate similarities between broilers and laying hens. Both breast meat yield and hen-day egg production increased linearly with PS while feed efficiency was affected in a quadratic fashion. The optimum estimated inclusion concentration to maximize feed efficiency was 25 and 26% for broilers and laying hens respectively, which is remarkably similar. Both feed intake and hen-day production responded in a linear fashion to PS concentration in laying hens. Thus, the change in feed efficiency could be a response only to the increased feed intake. However, feed efficiency showed a quadratic effect, suggesting an effect of the starch type in addition to feed intake. In broilers, low concentration levels of PS improved gain in broilers under equal feed intake, maximizing efficiency. These results indicate that PS produces a shift in metabolism towards production. However, as the rate or extent of digestion decreases, nutrient levels could be a limiting factor, resulting in reduced performance (e.g. decrease in broiler body weight as PS increases) or a compensatory response with increased feed intake as was observed in laying hens.

A number of the results support a metabolic effect of starch type; these include the decrease in carcass fat in broilers or the improvement in feather coverage in laying hens found with increasing diet PS. Research conducted in mammals has shown that SCFA have a regulatory effect on fat metabolism (Kondo et al., 2009; den Besten et al., 2013). SCFA activate fatty acid oxidation while lipogenesis is inhibited. The concentration of SCFA in the caeca of broilers showed a linear increase with PS concentration, and thus it could explain the observed shift in energy partitioning with PS. Insulin could also be a likely mediator of the observed effects. Simon et al. (2000) described the differences between two chicken lines selected for divergent abdominal fat deposition and the subsequent generations. They found that the main difference between lines was the glucose-insulin balance. Insulin was measured in different conditions and times, and it showed that the fat-line birds had a higher insulin concentration than the lean birds particularly after a glucose test. As more rapidly digested starch should promote higher insulin release, it is possible that the shift in energy partitioning observed in this study is the response to insulin concentration. Unfortunately, these concentrations were not measured and future research should include monitoring insulin.

The effect on feather pecking is more difficult to understand. In the laying hen experiment, satiety was not increased with PS, although this could be the result of the relative reduction of total starch in the diets as PS concentration increased and the resulting linear increase in feed intake with PS. Even though it could be argued that the improvement in feather cover could be the result of the increased feed intake observed with PS, feeding behaviour showed that there was no treatment effect on total time spent at the feeder. This suggests that diet composition impacted the motivation to feather peck. Meyer et al. (2013) reported a link between feather pecking and fermentation metabolites, indicating that protein fermentation was associated with lower feather pecking. As the proportion of protein increased with increased PS due to the lower total starch in PS, it is possible that more protein was being fermented. However, ileum pH in laying hens decreased as PS concentration increased, suggesting that there was enough starch or fibre remaining at the ileum level to be the primary energy source of the bacterial population, resulting in protein use for growth instead or energy. Alternatively, coarse fibre has proven to reduce feather pecking by reducing passage rate through the gizzard or by increasing satiety (Rodenburg et al., 2013). It is possible that a combination of gizzard development in middle ranges of PS inclusion and increased feed intake, which would activate nutrient sensing and

mechanical dilation receptors, could be reducing the motivation to feather peck. Alternatively, feather pecking incidence has been shown to be correlated with corticosterone concentrations (El-lethey et al., 2001). Stress was not measured in this study, but the feather cover improvement could be the results of a positive effect of PS on stress levels.

8.4 Digestive tract and activation of the ileal brake

Starch type affected digestive tract morphology and physiology. However, the effects observed in broilers and laying hens differed in a number of respects. This could be the result of the previously mentioned differences between trials such as differing bird types. Broilers were exposed to the experimental diets from day 0 while laying hens grew on age-appropriate commercial diets, starting the experiment at the age of 26 weeks. Thus, not only age and maturity of the digestive tract differed, but also laying hens had to go through an adaptation process to the new diets. Tissue collections occurred after four weeks of exposure in broilers while laying hens were exposed for 20 weeks, allowing a longer adaptation time. Finally, housing type could have affected our observations in the digestive tract since broilers were housed on floor pens, which allow them access to straw and excreta while laying hens were housed in conventional cages. Thus, other than feathers, no element other than the feed could have been ingested.

Both trials showed an increase in jejunum and ileum digesta content with PS, as well as elongation and higher empty weight of the ileum section with PS. Diets were formulated to be identical other than starch source. However, as diet PS increased, a larger amount of undigested material reached the distal portions of the digestive tract as shown by starch digestibility data in Chapter 5. In these digestive tract sections, as well as the caeca, the undigested materials are exposed to the fermenting capacity of the microbiome. This process results in the production of SCFA, in addition to other fermentation products. SCFA and the presence of fats and carbohydrates in the distal small intestine can promote L-cell activation, which among other products releases GLP-2. This peptide belongs to the glucagon superfamily and has been shown to promote intestinal mucosal growth (Scott et al., 1998; Thulesen et al., 1999). Butyrate has also shown to produce similar effects in the digestive tract tissue. Thus the increase in empty weight and length of the ileum could be the result of the fermentation capacity of the bacteria residing in the jejunum, ileum and caeca of chickens. Similar effects have been reported in the pig colon

when fed diets that included resistant starch (Bird et al., 2007). Fermentation in mammals occurs mostly in the large intestine, making it the target site for the physiological effects in contraposition to chickens where the ileum and caeca are the likely targets. An alternative explanation would be the increase in muscular development due to the increase digesta bulk with PS. Histological collections could determine some of these points in future research.

In terms of digesta content, broilers showed a linear increase with PS only in crop, jejunum and ileum digestive contents, while laying hens showed a response of content to dietary PS in crop, gizzard, duodenum, jejunum and ileum, with only crop content changing quadratically while the digesta contents in the remaining sections increased linearly with PS. Broiler feed intake was not affected by dietary PS, and thus the increase in contents in the small intestine is at least partially due to a higher amount of undigested material due to higher PS content. The increase in crop content, on the other hand, suggests a decrease in passage rate due to a reduction in gizzard emptying or differences in the quantity of feed ingested per meal. In contrast with the broiler trial, laying hen feed intake linearly increased with PS, which could explain the linear increase in gizzard digesta weight. The linear increase in small intestine digesta weight could be the result of a combination of more undigested material, like it was observed in broilers, in addition to the increased feed intake. The quadratic change observed in crop content, however, correlated with the weight of the empty gizzard, suggesting again a reduction in passage rate in the proximal section of the digested tract in the mid-ranges of PS. Although both broiler and laying hen digestive tract results suggest a regulation of passage rate associated with PS content, the results of laying hen passage rate indicate diet had no effect on it. It could be that a reduction in passage rate in the proximal digested tract is associated with a faster passage rate in the small intestine due to gizzard development and associated improved nutrient digestibility. Alternatively, Chen et al. (1997) showed that while low concentrations of PYY produce a brake via reduced gastrointestinal motility in rats, high concentrations have the opposite effect and that this difference is mediated through the activation of two different PYY receptors.

Digestive tract pH data also differed between broilers and laying hens. While broilers only demonstrated a quadratic effect of PS on crop digesta pH that suggests a saturation of the crop bacterial population, laying hens reacted with a linear decrease in digesta pH, both in crop and ileum as PS increased. These differences may indicate different stages of an adaptation process

to the experimental diets. Broilers were fed three different diets during 28 d and although there is undoubtedly good adaptation of the bacterial community, the length of time may not be enough to reach its maximum potential. Laying hens were exposed to the same diets for four months, allowing for a longer adaptation time, which resulted in a linear decrease of pH both in crop and ileum. The lack of pH change in caeca suggests that most of the PS is completely fermented or digested before entering the caeca.

A completely different effect of PS on PYY and GLP-1 concentrations was found between broilers and laying hens. Both ELISA and gene expression results in broilers suggest no effect of PS on the activation of L-cells. Laying hen ELISA results, on the contrary, indicate a linear increase of GLP-1 serum concentrations and a quadratic response of PYY concentrations with a maximum at 34% PS. Although it is true that laying hen feed intake increased with PS, an increase in digesta content in jejunum and ileum sections occurred in both bird types, and in similar amounts. The difference in jejunum digesta content between 100 and 0% PS was 6 and 5 g for broilers and laying hens respectively, while ileum digesta increased by 3 and 4 g respectively. Thus, it is unlikely that feed intake could be the reason for the difference in response. Alternatively, selection for performance might have resulted in a different sensitivity of the enteroendocrine cells to luminal nutrients between bird types, resulting in different responses.

Another interesting point is that although PYY concentrations were similar between bird types (broiler = 362 vs. laying hen = 427 pg/mL), although higher than those reported by Lee et al. (2017; average serum PYY = 156 pg/mL), GLP-1 serum concentrations differed remarkably between them (broiler = 1652 vs. laying hen = 698 pg/mL). No GLP-1 serum concentrations in chicken have been reported before, but the levels reported here are higher than those reported for humans or dogs, which are usually in the range of 8 to 40 pmol/L (Massimino et al., 1998; Wachters-Hagedoorn et al., 2006; Vollmer et al., 2008). This difference might be because mammals eat discrete meals while domestic chickens eat small meals frequently, which results in a constant stimulation of the enteroendocrine cells. This could result in resistance or desensitization to these neuropeptides, and no or reduced ileal brake mechanism in chickens, possibly explaining the the lack of passage rate or feed intake response in laying hens to PYY and GLP-1.

8.5 Feeding behaviour

Broiler feed intake did not differ between treatments, while laying hen feed intake increased as PS increased. Broilers eat until they reach a point close to the maximum capacity of the digestive tract. That means that their ability to adjust feed intake is small, which could explain the lack of difference observed in feed consumption between treatments. Some strains of laying hens on the other hand, have at least some ability to change their feed intake in response to the characteristics of the feed. The increase observed in feed intake with PS could be in response to a lower AME value in higher PS diet due to a reduction in starch content. Regardless of differences in feed intake, changes in satiety could be observed not only by measuring feed intake, but also by looking at feeding patterns, as satiety is associated with a longer time between meals. As feed intake was not measured in each visit, it was assumed that each visit to the feeder involved feed consumption, although, as shown by the laying hen data, this might not always be the case.

Broiler feeding behaviour was consistent with the lack of effect found on broiler feed intake. Although statistical differences were found between dietary treatments, differences followed a random pattern, suggesting little to no effect of PS concentration. The average feeding time per hour was very similar between dietary treatments and between genders. Data shows that broilers exhibit compensatory feeding at the beginning of the day in order to refill the digestive tract after the night period. However, no evidence of increase in feeding time is observed at the end of the day in this study. This could suggest a digestive tract already at full capacity or a different rate of feed intake. As no night feeding was observed, it could support a higher rate of feed intake at the end of the day, as the lack of night feeding indicates that the amount of feed in their digestive tracts was enough to maintain content in the tract through the night. The data also showed gender differences consistent with feed intake. Males grew larger than females in the same amount of time, implying a higher metabolic demand of their bodies. Not only feed intake was higher in males, but also they exhibit a different feeding pattern, indicative of higher hunger levels. Males visited the feeder more than females in order to satisfy their needs.

Meanwhile, laying hen feeding behaviour differed from day to night time. Feeding behaviour was statistically identical between treatments during the photoperiod despite differences in feed intake. All hens showed both compensatory feeding at the beginning of the

day and anticipatory feeding (cropping up) prior to the scotoperiod. During the scotoperiod, however, two parameters were affected by dietary treatment: number of visits to the feeder and when these visits started after the lights were turned off. The number of night visits to the feeder increased with PS. Also, the higher the dietary PS concentration, the sooner the first night visit to the feeder occurred. Still, total night feeding time showed no differences between treatments. Thus, contrary to the hypothesis, the data indicate that the higher the PS concentration, the higher the level of hunger, as these were the birds that fed during the night. Total time spent at the feeder, both during the photo and the scotoperiods, was the same among treatments, implying that the rate of feeding differed between treatments, and that not all the time spent at the feeder involved feeding or not every feeder visit involved feeding. As feed intake increased with PS concentration, it means that hens in the lower concentrations of PS spent time at the feeder performing activities other than feeding.

Feeding behaviour data were collected based on the hypothesis that the presence of PS in the distal small intestine would result in the release of PYY and GLP-1, which would slow digesta passage, increase satiety and alter feeding patterns. It was confirmed that the presence of starch along the digestive tract increased as dietary PS concentration increased. The results indicate that although the serum concentrations of GLP-1 and PYY were high, and at least in laying hens responded to dietary concentration of PS, when compared to reported concentrations in mammals, satiety was not activated. What is more, laying hens showed the opposite pattern to that of the hypothesis, exhibiting more signs of increased hunger during the night time as PS concentration increased. Feeding behaviour showed to be more consistent with feed intake measurements, as the lack of differences in broiler feed intake was reflected by no or random differences in feed intake, while laying hens, whose feed intake increased with PS concentration, showed signs of increased hunger at night.

8.6 Conclusions

In conclusion, the positive effects of formulating diets that include starches with differing digestion rates have been successfully confirmed using semi-purified starch. What is more, it was shown that the positive effect is more extensive than simply improving feed efficiency in broilers. Evidence presented here shows that a shift in energy partitioning in broilers from fat to muscle occurs and it appears to depend on the percentage of the more slowly digested starch

source present in the diet. However, although the insulin-glucose balance, as well as SCFA concentrations, could be potential mediators, more research is needed to assess them. In addition, this project went beyond the effects of rate and extent of starch digestion in broilers and also revealed that laying hens respond positively to the combination of starches with differing digestion rates, both in terms of egg production and feed efficiency.

The physiological parameters measured indicate that an adaptation of the digestive tract and its microbiome occurs to respond more efficiently to the presence of more slowly digestive sources in the diets. Also, the evidence does not support the activation of the ileal brake. Although PYY and GLP-1 concentrations appear to be high when compared to mammals, the lack of previous publications of these values in chickens only allows for speculation. If indeed these concentrations are high, it confirms L-cell activation by either the presence of glucose or SCFA in the jejunum or ileum lumen, or merely due to the fact that digesta is always present along the digestive tract. However, the lack of response in passage rate and the fact that feeding behaviour showed no effect or the opposite to that expected by the activation of the ileal brake suggest that chickens might not respond to this mechanism, and more research is needed to understand other potential mechanisms involved.

8.7 Future research

Future research should focus on determining the physiological mediator of the shift in energy partitioning observed in this study. Understanding how PS or other sources of slowly digested starch affect this metabolism will help to accurately formulate diets in the future to reach the desired effect. In addition, a histological analysis explaining the morphological changes observed in the digestive tract, particularly the ileum, could determine if the changes in empty weights are due to a trophic effect on the mucosa or/and thickening of the muscle layer due to the increasing amount of digesta content. Finally, the morphological and physiological effects reported here should also be confirmed with the use of practical diets and other sources of more slowly digested starch.

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APPENDIX A

In order to evaluate early indicators of ileal brake activation in broilers (Chapter 5), an additional tissue collection was performed at 14 d of age that included digestive tract removal and in situ pH measurement. In addition, absolute and relative organ weights at 28d and absolute digestive tract data from 28 d of age collection are presented here.

Table A.1. Effect of the proportion of dietary wheat and pea starch on crop, ileum and caeca contents pH of broilers at 14 d of age

	Diets						Regression			SEM
	0[1]	**20**	**40**	**60**	**80**	**100**	***P* value**	**R^2**	**Equation**	
Crop	5.5	5.4	5.2	5.3	5.3	5.2	NS	-	-	0.07
Ileum	6.9	6.5	6.7	6.6	6.6	6.5	NS	-	-	0.06
Caeca	6.4	6.5	6.6	6.3	6.3	6.2	L=0.018	0.06	y = -0.003x + 6.5	0.04

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.
Data was applied natural logarithm transformation; *P* value: indicates Regression *P* value; Q: quadratic regression; SEM: Pooled standard error of the mean; Number of replications = 4.

Table A.2. Effect of the proportion of dietary wheat and pea starch on digestive tract empty weights, contents and small intestine length of broilers at 14 d of age

	Diets						Regression			SEM
	0[1]	**20**	**40**	**60**	**80**	**100**	***P* value**	**R^2**	**Equation**	
Body weight (gr)	498	489	496	512	518	500	NS	-	-	13.4
Empty weights (g)										
Crop	3.0	3.0	3.3	3.1	3.2	3.0	NS	-	-	0.08
Proventriculus	3.0	2.9	3.0	3.0	3.3	3.2	L=0.005	0.08	$y = 0.003x + 2.9$	0.04
Gizzard	11.5	10.8	11.3	11.8	12.2	11.4	NS	-	-	0.16
Duodenum	6.0	6.0	5.9	6.1	5.9	6.0	NS	-	-	0.10
Jejunum	9.3	9.6	9.8	10.5	10.7	9.5	Q=0.026	0.08	$y = -3_x10^{-4}x^2 + 0.04x + 9.1$	0.15
Ileum	7.0	7.4	7.4	8.0	7.9	8.0	L<0.001	0.13	$y = 0.01x + 7.1$	0.10
Caeca	2.2	2.4	2.3	2.5	2.6	2.7	L<0.001	0.14	$y = 0.005x + 2.2$	0.05
Length (cm)										
Duodenum	22	22	22	23	23	23	L=0.012	0.07	$y = 0.01x + 22$	0.2
Jejunum	51	54	51	58	55	57	L<0.001	0.12	$y = 0.06x + 52$	0.6
Ileum	48	51	49	56	53	54	L<0.001	0.12	$y = 0.06x + 49$	0.6
Caeca	18	19	18	20	19	19	L=0.002	0.09	$y = 0.02x + 18$	0.2
Total	139	145	140	157	150	154	L<0.001	0.16	$y = 0.1x + 140$	1.3
Contents (g. as is basis)										
Crop	4	7	10	7	8	7	Q=0.025	0.09	$y = -0.001x^2 + 0.1x + 4$	0.5
Proventriculus	0.4	0.4	0.6	0.4	0.6	0.4	NS	-	-	0.05
Gizzard	8	7	7	9	10	10	L=0.002	0.10	$y = 0.02x + 7$	0.3
Duodenum	1.2	0.8	1.2	1.4	1.4	1.4	L=0.037	0.05	$y = 0.004x + 1.0$	0.07
Jejunum	5.5	5.6	5.7	6.7	6.6	6.9	L=0.004	0.09	$y = 0.02x + 5.4$	0.18
Ileum	4.6	4.6	5.6	6.4	5.7	5.5	Q=0.008	0.15	$y = -4_x10^{-4}x^2 + 0.05x + 4.3$	0.15
Caeca	1.3	1.7	1.6	1.8	2.0	1.9	L=0.025	0.05	$y = 0.005x + 1.4$	0.08

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.
P value: indicates Regression *P* value; L: linear regression; NS: Not significant; SEM: Pooled standard error of the mean; Number of replications = 4.

Table A.3. Effect of the proportion of dietary wheat and pea starch on digestive tract empty weights, contents and small intestine length of broilers at 14 d of age as a percentage of body weight

	Diets						Regression			SEM
	0[1]	20	40	60	80	100	*P* value	R^2	Equation	
Body weight (gr)	498	489	496	512	518	500	NS	-	-	13.4
Empty weights (% of body weight)										
Crop	0.60	0.60	0.66	0.60	0.61	0.61	-	-	-	0.014
Proventriculus	0.60	0.59	0.61	0.59	0.63	0.64	L=0.013	0.06	$y = 5_x10^{-4}x + 0.59$	0.007
Gizzard	2.31	2.22	2.28	2.32	2.38	2.28	-	-	-	0.027
Duodenum	1.21	1.24	1.20	1.20	1.13	1.20	-	-	-	0.020
Jejunum	1.87	1.97	1.99	2.05	2.07	1.90	Q=0.031	0.06	$y = -6_x10^{-5}x^2 + 0.007x + 1.86$	0.027
Ileum	1.42	1.51	1.51	1.56	1.53	1.61	L=0.008	0.07	$y = 0.002x + 1.44$	0.022
Caeca	0.44	0.49	0.47	0.49	0.50	0.54	L=0.005	0.08	$y = 8_x10^{-4}x + 0.44$	0.010
Length (cm/100 g of BW)										
Duodenum	4.5	4.5	4.5	4.6	4.5	4.6	-	-	-	0.06
Jejunum	10.4	11.1	10.4	11.5	10.7	11.5	-	-	-	0.15
Ileum	9.8	10.5	9.9	11.1	10.3	10.8	L=0.049	0.04	$y = 0.008x + 10.0$	0.14
Caeca	3.5	3.9	3.7	3.9	3.8	3.9	-	-	-	0.06
Total	28.2	30.0	28.5	31.0	29.2	30.8	-	-	-	0.36
Contents (% of body weight)										
Crop	0.70	1.42	1.92	1.37	1.54	1.38	Q=0.030	0.08	$y = -2_x10^{-4}x^2 + 0.03x + 0.81$	0.101
Proventriculus	0.08	0.07	0.13	0.08	0.10	0.08	-	-	-	0.010
Gizzard	1.68	1.44	1.45	1.72	1.90	1.89	L=0.003	0.09	$y = 0.004x + 1.49$	0.045
Duodenum	0.24	0.17	0.25	0.28	0.27	0.28	-	-	-	0.015
Jejunum	1.11	1.15	1.13	1.31	1.28	1.40	L=0.010	0.07	$y = 0.003x + 1.10$	0.036
Ileum	0.92	0.96	1.13	1.26	1.09	1.10	Q=0.006	0.14	$y = -7_x10^{-5}x^2 + 0.009x + 0.89$	0.027
Caeca	0.27	0.36	0.32	0.35	0.39	0.37	-	-	-	0.017

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.
P value: indicates Regression *P* value; L: linear regression; NS: Not significant; SEM: Pooled standard error of the mean; Number of replications = 4.

Table A.4. Effect of the proportion of dietary wheat and pea starch on digestive tract empty weights, contents and small intestine length of broilers at 28 d of age

	Diets						Regression			SEM
	0[1]	**20**	**40**	**60**	**80**	**100**	***P* value**	**R^2**	**Equation**	
Body weight (g)	1712	1705	1697	1729	1588	1636	L=0.042	0.04	y = -1x + 1728	16.8
Empty weights (g)										
Crop	5.4	5.6	5.6	5.5	5.9	6.1	-	-	-	0.12
Proventriculus	6.1	6.3	6.6	6.3	6.2	6.7	-	-	-	0.12
Gizzard	17.8	17.3	19.5	18.4	16.4	17.6	-	-	-	0.29
Duodenum	9.9	10.0	10.9	10.4	9.8	9.8	-	-	-	0.19
Jejunum	21.9	22.9	22.9	22.8	22.5	23.3	-	-	-	0.41
Ileum	15.9	16.1	17.2	17.5	16.4	17.0	-	-	-	0.29
Total SI	48	49	51	51	49	50	-	-	-	0.4
Caeca	5.5	5.4	6.1	5.8	5.8	5.9	-	-	-	0.11
Colon	1.47	1.48	1.50	1.55	1.50	1.67	-	-	-	0.028
Length (cm)										
Duodenum	27	28	27	29	28	27	-	-	-	0.3
Jejunum	63	67	65	68	67	68	L=0.032	0.05	y = 0.04x + 64	0.7
Ileum	62	67	68	70	67	70	L=0.016	0.06	y = 0.06x + 65	0.8
Total SI	152	162	160	168	163	165	L=0.020	0.06	y = 0.1x +156	0.1
Caeca	26	27	27	27	27	28	-	-	-	0.3
Colon	4.4	4.8	4.5	4.5	4.4	4.6	-	-	-	0.08
Contents (g. as is basis)										
Crop	8	12	11	16	18	24	L<0.001	0.16	y = 0.2x + 8	1.3
Proventriculus	0.8	1.1	1.1	0.8	1.7	1.7	-	-	-	0.17
Gizzard	12	8	13	12	9	11	-	-	-	0.7
Duodenum	3.9	3.7	3.4	3.6	3.9	3.5	-	-	-	0.11
Jejunum	14	16	15	19	21	20	L<0.001	0.22	y = 0.07x + 14	0.5
Ileum	13	14	15	18	16	16	L=0.008	0.07	y = 0.04x + 13	0.5
Total SI	31	33	34	40	41	40	L<0.001			0.4
Caeca	4.5	3.9	4.3	3.8	5.0	4.7	-	-	-	0.19
Colon	0.8	1.2	1.0	0.9	0.9	1.0	-	-	-	0.05

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.
P value: indicates Regression *P* value; L: linear regression; NS: Not significant; SEM: Pooled standard error of the mean; Number of replications = 4.

Table A.5. Effect the proportion of dietary wheat and pea starch on heart, liver and pancreas weight of broilers at 14 and 28 d of age

	Diets						Regression			SEM
	0[1]	20	40	60	80	100	*P* value	R^2	Equation	
14 d of age (g – as is basis)										
Heart	3.5	3.0	3.0	3.3	3.6	3.3	NS	-	-	0.08
Liver	21.5	20.5	21.0	21.4	20.7	20.5	NS	-	-	0.33
Pancreas	1.9	1.8	1.9	2.0	2.0	1.9	NS	-	-	0.03
28 d of age (g – as is basis)										
Heart	9.2	9.7	9.8	9.6	9.4	9.2	NS	-	-	0.17
Liver	57	60	58	57	55	54	NS	-	-	0.8
Pancreas	3.8	3.8	4.0	3.8	3.6	3.9	NS	-	-	0.07
14 d of age (% of body weight)										
Heart	0.64	0.63	0.61	0.65	0.65	0.66	NS	-	-	0.015
Liver	4.3	4.2	4.3	4.2	4.0	4.1	NS	-	-	0.06
Pancreas	0.38	0.36	0.39	0.40	0.40	0.38	NS	-	-	0.007
28 d of age (% of body weight)										
Heart	0.54	0.57	0.58	0.56	0.59	0.57	NS	-	-	0.010
Liver	3.3	3.5	3.4	3.3	3.5	3.3	NS	-	-	0.04
Pancreas	0.22	0.23	0.24	0.27	0.23	0.24	NS	-	-	0.004

[1] Diets denominations indicate the percentage of pea starch in the starch fraction of the diet.
Data was applied natural logarithm transformation; *P* value: indicates Regression *P* value; Q: quadratic regression; SEM: Pooled standard error of the mean; Number of replications = 4.

APPENDIX B

During digestive tract collections in laying hens (Chapter 6), as well as at the end of the experiment, individual body weights were recorded. Body weights of individuals chosen for tissue collections showed that PS concentration resulted in a quadratic trend, while the quadratic effect in body weights was confirmed by the body weights recorded at the end of the experiment. In consequence, digestive tract data was also analyzed as a percentage of body weight.

Table B.1. Effect of the proportion of dietary wheat and pea starch on the relative digestive tract empty weight and digesta content, and small intestine length of laying hens at 46 weeks of age

	Diets						Regression		SEM
	0[1]	20	40	60	80	100	*P* value	R^2	Equation
Body weight (g)	1678	1681	1733	1854	1756	1729	NS	-	-	17.6
Empty weight (% of body weight)										
Crop	0.40	0.42	0.41	0.43	0.43	0.44	NS	-	-	0.011
Proventriculus	0.35	0.34	0.36	0.34	0.34	0.36	NS	-	-	0.508
Gizzard	0.61	0.59	0.70	0.69	0.63	0.58	Q=0.012	0.09	$y = -3_x10^{-5}x^2 + 0.003x + 0.59$	0.002
Duodenum	0.51	0.52	0.49	0.45	0.51	0.52	Q=0.034	0.04	$y = 1_x10^{-5}x^2 - 0.002x + 0.52$	0.008
Jejunum	0.95	0.91	0.85	0.79	0.93	0.98	Q<0.001	0.11	$y = 5_x10^{-5}x^2 - 0.005x + 0.96$	0.015
Ileum	0.78	0.83	0.75	0.75	0.83	0.88	Q=0.020	0.08	$y = 3_x10^{-5}x^2 - 0.002x + 0.80$	0.012
Caeca	0.43	0.43	0.41	0.41	0.41	0.43	NS	-	-	0.008
Colon	0.12	0.12	0.11	0.10	0.11	0.11	NS	-	-	0.003
Length (cm/100 g of BW)										
Duodenum	1.42	1.45	1.42	1.34	1.42	1.42	NS	-	-	0.017
Jejunum	3.20	3.20	3.06	2.85	3.08	3.29	Q=0.009	0.07	$y = 1_x10^{-4}x^2 - 0.01x + 3.29$	0.042
Ileum	3.00	3.00	2.83	2.76	2.96	3.08	Q=0.028	0.04	$y = 9_x10^{-5}x^2 - 0.009x + 3.03$	0.042
Caeca	1.44	1.42	1.45	1.46	1.49	1.44	NS	-	-	0.020
Colon	0.25	0.24	0.23	0.23	0.24	0.23	NS	-	-	0.005
Total	9.30	9.31	8.99	8.63	9.19	9.46	Q=0.050	0.04	$y = 2_x10^{-4}x^2 - 0.02x + 9.44$	0.110
Content (% of body weight)										
Crop	0.76	1.07	1.10	1.33	0.79	0.64	Q=0.019	0.06	$y = -9_x10^{-5}x^2 + 0.02x + 0.64$	0.097
Proventriculus	0.05	0.03	0.05	0.04	0.05	0.04	NS	-	-	0.003
Gizzard	0.15	0.18	0.17	0.16	0.21	0.20	NS	-	-	0.012
Duodenum	0.15	0.18	0.17	0.16	0.21	0.20	L=0.002	0.08	$y = 5_x10^{-4}x + 0.16$	0.006
Jejunum	0.63	0.73	0.76	0.71	0.87	0.86	L<0.001	0.10	$y = 0.002x + 0.65$	0.022
Ileum	0.44	0.48	0.52	0.52	0.56	0.63	L<0.001	0.12	$y = -0.002x + 0.43$	0.017
Caeca	0.17	0.20	0.17	0.19	0.16	0.19	NS	-	-	0.008
Colon	0.042	0.044	0.042	0.039	0.044	0.050	NS	-	-	0.0027

[1] Diet denominations indicate the percentage of pea starch in the starch fraction of the diet.

L: linear regression; Q: quadratic regression; NS: Not significant; SEM: Pooled standard error of the mean; Number of replications = 6.